GlycoBioTec 2023

January 17-19, 2023 / Harnack Haus / Berlin, Germany

Dear colleagues and participants

The Max Planck Institute for Dynamics of Complex Technical Systems Magdeburg is very pleased to welcome you to Berlin for the next iteration of the GlycoBioTec conference.

With the Corona pandemic, it took us four years to host this meeting again after the very successful second edition in 2019, which brought together about 180 participants from both academia and industry. After receiving very encouraging feedback and suggestions for new topics, we have decided to develop this platform further for the exchange of ideas of opinion leaders, pioneers, experts, professionals and newbies in the field of glycobiotechnology. Therefore, as part of the last meeting, we have also established a scientific committee to support the selection of topics, speakers and poster presentations.

In the coming days, we kindly invite you to present and discuss the newest trends and cutting-edge research on the frontiers of glycobiotechnology. As in the previous years, the sessions cover a wide range from glycosylation and protein function, synthesis of glycoconjugates, glycans and nucleotide sugars, cell-based and cell-free glycoengineering of biopharmaceuticals, synthesis and function of human milk oligosaccharides, glycoanalytics, glycoimmunology and glycobioinformatics

We hope you will benefit from participation and enjoy the talks and discussions as well as your stay at the Harnack house in Berlin.

Yours sincerely,

Udo Reichl and Thomas Rexer

VENUE

Harnack Haus
Ihnestr. 16-20
14195 Berlin
Tel.: +49 30 8413-3800

WLAN: HH-Guest
Password: Oxygen.08

The Harnack Haus, History:

The Harnack House was built in 1929 to provide guest accommodation and a conference venue for the Kaiser Wilhelm Society, the Max Planck Society's predecessor. In the 1930s it was used as a club for international science and as a social venue in the German capital. Scientists including many Nobel laureates, artists, politicians and industrial magnates visited the Harnack House. After the Second World War, the Harnack House was used as an officers' club until the withdrawal of the Allies from Berlin in 1994. It was subsequently handed over to the Max Planck Society.

Today, the Harnack House is again a meeting place for the international scientific community of the Max Planck Society and its guests from all over the world.

Registration desk

The registration desk is open on Monday (6:30 PM – 9:00 PM), Tuesday (8:00 AM - 10:00 AM) and Wednesday (8:30 AM – 9:00 AM). Please feel free to come here if you have any questions or directly approach the organizing committee.

In urgent cases, please message +49 176 83536108 (Thomas Rexer, Organizing Committee).

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MEETING ROOM

A meeting room ("Ruska") is bookable for all GlycoBioTec participants for business meetings during the conference. The Ruska meeting room is located on the first floor of the Harnack House and is accesible through the staircase at the main entrance.

A slot in the meeting room can be booked via the following link:

Passwort: **ruska@gbt**

Please do not forget to save the file, when booking this room.

EINSTEIN LOUNGE

The Einstein Lounge

The Einstein Lounge offers the appropriate setting for a convivial end with intensive discussions about lecture topics and future projects. So, please join us here for a drink and feel free to network and connect with colleagues!

The curved bar is a relic from the time when the Harnack House was still the officers' casino of the US Army, which was stationed in Berlin until 1994, and you could only pay here with dollars. It contributes to the special lounge atmosphere. The Einstein Lounge owes its name to probably the most famous guest of the Harnack House: Albert Einstein. He gave two lectures at Harnack House at the beginning of the 20th century.

ORGANIZING COMMITTEE

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KEYNOTE SPEAKERS

Horst Bierau

Merck Serono S.p.A., Rome, Italy

Glycosylation challenges in biopharmaceutical manufacturing

Horst Bierau is a Senior Scientific Advisor and heads the CMC Science & Intelligence group in the Healthcare division of Merck. He has 20 years of experience in biopharmaceutical product development for a variety of disease indications and his research interests focus on CMC development.

Lai-Xi Wang

University of Maryland, College Park, USA

Antibody glycosylation and site-specific antibody-drug conjugates

Lai-Xi Wang is Professor of Chemistry and Biochemistry at the University of Maryland, College Park. He received his PhD from Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. After postdoctoral studies in glycobiology and molecular biology at Johns Hopkins University and MIT, respectively, he joined the faculty of University of Maryland as an assistant professor in 2000 and was promoted to full professor in 2009. His research focuses on development of new chemoenzymatic methods for glycoprotein synthesis and antibody glycoengineering and on their applications for functional studies and drug discovery. The site-specific Fc glycan remodeling method developed by his team enables a quick access to various homogeneous antibody glycoforms for immunological studies. More recently, his team has developed a general and efficient chemoenzymatic approach for site-specific antibody labeling and bioconjugation. It provides a platform technology for producing homogeneous antibody-drug conjugates and antibody-ligand conjugates for therapeutic development.

Katja Parschat

Chr Hansen HMO GmbH, Rheinbreitbach, Germany

Human milk oligosaccharides: New infant formula ingredients with diverse health benefits

Katja Parschat is Head of the R&D department at the Chr. Hansen HMO GmbH that is part of the Danish bioscience company Chr. Hansen A/S. The Chr. Hansen HMO GmbH focusses on the development of economic production processes and the production of Human Milk Oligosaccharides (HMOs). The R&D team in Germany engineers E. coli to highly productive HMO producers and develops efficient fermentation processes. In addition, a team of scientists in Denmark investigates beneficial health effects of HMOs in in-vitro, in-vivo and in clinical studies. Katja Parschat holds a PhD in molecular microbiology from the Carl von Ossietzky University in Oldenburg. After a post-doc period at the University of Münster she started working in the field of HMO process development and HMO applications.

Kaspar Locher

Department of Biology, ETH Zurich, Zurich, Switzerland

Structure and mechanism of ER-luminal enzymes involved in protein glycosylation

Kaspar Locher studied Chemistry/Biochemistry at the Swiss Federal Institute of Technology (ETH) Zurich. He obtained a PhD in Biochemistry from the University of Basel in 1998, where he pursued studies with Prof. J. Rosenbusch at the Biozentrum. From 1999-2003, he was a post-doctoral researcher with Prof. Douglas Rees at the California Institute of Technology. In 2003, he joined ETH Zurich as an Assistant Professor and was promoted to Full Professor of Molecular Membrane Biology in 2013. Locher is interested in the structural and mechanistic studies of membrane proteins. His research focus includes the investigation of glycosyltransferases that are part of the protein N-glycosylation machinery. His goal is to assemble molecular "movies" of these essential biochemical reactions by combining high resolution protein structures with biochemical and biophysical studies. In addition to generating insight into the mechanism of physiologically important reactions, his studies provide opportunities for future drug discovery.

Peter Seeberger

Max Planck Institute of Colloids and Interfaces, Potsdam, Germany

Automated glycan assembly as basis for life science and material science applications

Peter H. Seeberger studied chemistry in Erlangen (Germany) and completed a PhD in biochemistry in Boulder (USA). After a postdoctoral fellowship at the Sloan-Kettering Cancer Center Research in New York he advanced to tenured Firmenich Associate Professor of Chemistry at MIT. After six years as Professor at the Swiss Federal Institute of Technology (ETH) Zurich he assumed positions as Director at the Max-Planck Institute for Colloids and Interfaces in Potsdam and Professor at the Free University of Berlin in 2009. Since 2021, he is a Vice President of the German Research Foundation (DFG) the main funding body in Germany. He is a member of the governing body of the Max-Planck Society (“Senate”) and the Veterinary University (TiHo) Hannover (“Stiftungsrat”). Professor Seeberger’s research has been documented in over 630 peer-reviewed journal articles, five books, more than 50 patents, and more than 900 invited lectures. This work was recognized with more than 35 international awards. Seeberger has been the Editor-in-Chief of the platinum open access Beilstein Journal of Organic Chemistry and serves on the editorial advisory boards of many other journals. He is a co-founder of the Tesfa-Ilg “Hope for Africa” Foundation. The research in the Seeberger laboratory has given rise to several successful companies in the USA, Switzerland, Denmark and Germany.

Salome Pinho

i3S - Institute for Research and Innovation in Health University of Porto, Porto, Portugal

Glycans at the frontiers of chronic inflammation, autoimmunity and cancer: mechanisms and clinical implications

Salomé Pinho received her D.V.M. from the University of Porto in 2004 and developed her PhD research at the Institute of Molecular Pathology and Immunology of Univ. Porto (IPATIMUP) and at Boston Medical School, MA, USA from 2006 to 2009. She performed her postdoctoral work at University of Porto in the cancer glycobiology field. At present, she is Principal Investigator at the Institute for Research and Innovation in Health (i3S) at University of Porto and Professor at Faculty of Medicine of University of Porto, Portugal. Her research activity is focused on the understanding of the role of post-translational modifications by glycosylation in the regulation of key proteins´ functions involved in cancer, chronic inflammation and autoimmunity, envisioning potential clinical applications. She is author of several publications in international peer-reviewed journals (as first and senior author) including Nature Reviews Cancer, Oncogene, PNAS, Gastroenterology, Arthritis & Rheumatology; Cancer Immunology Research among others. She coordinates a multidisciplinary research group (Immunology, Cancer & GlycoMedicine) at i3S composed by Post-Docs, PhD students, Master students, young researchers and clinical researchers. She is the Principal Investigator of several national/international funded projects in the field of glycobiology, cancer and inflammatory diseases, including the coordination of Horizon Europe grants and ERC grants. She has been awarded with several prizes including the Young Investigator Award from the European Association for Cancer Research; the Glycobiology Significant Achievements award in 2020 by the American Society of Glycobiology and the Pfizer Award for Clinical Research in 2021.

SCIENTIFIC COMMITTEE

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Erdmann Rapp	glyXera GmbH
Udo Reichl	Max Planck Institute Magdeburg
Dietmar Reusch	Roche AG
Thomas Rexer	Max Planck Institute Magdeburg

PROGRAMME

PROGRAMME

TUESDAY, JANUARY 17TH, 2023

8:00 Registration

9:15 Opening remarks: Prof. Udo Reichl (Director MPI DCTS), Thomas Rexer (MPI DCTS)

Session 1: Glycosciences – Fundamentals and Advances in Clinical and Biopharmaceutical Research

Chair: Marcelo Guerin (Biocruces Bizkaia Health Research Institute)

9:30 **Keynote Lecture:**
Kaspar Locher, ETH Zurich, Switzerland
Structure and mechanism of ER-luminal enzymes involved in protein glycosylation

10:10 Miloslav Sanda, Georgetown University & Max Planck Institute for Heart and Lung Research, USA
Glycans of the SARS-CoV - 2 spike protein

10:30 Thomas Klaric, Yale School of Medicine & Genos, USA
Multiregional comparative N-glycomics reveals both spatial and phylogenetic gradients in mammalian brain N-glycome complexity

10:50 Ines Moreira, Hannover Medical School, Germany
Neolactotetraosylceramide is a novel biomarker for bladder cancer

11:10 Break with Coffee, Tea & Snacks

11:40 Timm Fiebig, Hannover Medical School, Germany
Fermentation-free and enzymes-based synthesis of vaccine antigens from Neisseria Meningitidis, Actinobacillus Pleuropneumoniae and Haemophilus Influenza

12:05 Marium Khaleque, University of Queensland, Australia
Differential *N*-Glycosylation site occupancy depends on distinct amino acid sequence features

12:25 **Lightning talks** (8 x 5 min)
Vladimir Kren, Pavla Bojarova, Lisa Wenzel, Olga Zaytseva , Charlotte Rossdam, Marco Albers, Brigitte-Carole Keumatio D., Roger Laine

13:05 Lunch

Session 2: Novel Emerging & Enabling Systems in Glycobiotechnology

Chair: Mattias Collin (Lund University)

14:30 **Keynote Lecture:**
Peter Seeberger, Max Planck Institute of Colloids and Interfaces, Germany
Automated glycan assembly as basis for life science and material science applications

15:10 Oren Moscovitz, Max Planck Institute of Colloids and Interfaces, Germany
Generation of glycan-specific nanobodies

15:35 Kai Hußnätter, RWTH Aachen, Germany
Towards automated enzymatic glycan synthesis with microgel immobilized glycosyltransferases in a countercurrent flow reactor

16:00 Break with Coffee, Tea & Snacks

16:30 Elli Makrydaki, Imperial College London, UK
SUGAR-TARGET: An immobilised enzyme cascade for targeted and bespoke glycosylation

16:50 Felix Löffler, Max Planck Institute of Colloids and Interfaces, Germany
Parallel synthesis of glycans by vapor coupling

17:10 **Lightning talks** (8 x 5 min)
Hannes Frohnmeyer, Alberto Alcalá, Tuan Hoang Son, Charles Williams, Myrna Bunte, Jennifer F. Alacorn, Edgar Gonzales-Rodriguez

17:50 Poster Session and Dine & Discuss – Food, beer, wine and soft drinks are included

20:00 Einstein Lounge

WEDNESDAY, JANUARY 18TH, 2023

Session 3: Synthesis and Function of Human Milk Oligosaccharides

Chair: Lothar Elling (RWTH Aachen)

9:00 **Keynote Lecture:**
Katja Parschat, Chr. Hansen HMO GmbH, Rheinbreitbach, Germany
Human milk oligosaccharides: New infant formula ingredients with diverse health benefits

10:10 Andrew McDonald, Trinity College Dublin, Ireland
Simulated symbioses: how human milk oligosaccharides influence the nascent gut microflora

10:30 Bernd Stahl, Danone Nutricia Research & Utrecht University, Netherlands
Human Milk: Oligo?Saccharides and more

10:55 Melda Karyelioglu, Canakkale Onsekiz Mart University, Turkey
Designing an in-vitro digestion model by using novel microbiome-associated enzymes to study glycan function

11:15 Break with Coffee, Tea & Snacks

11:45 Nils Banke, Glycom A/S part of DSM, Netherlands
Cell factory engineering and process design to make human milk oligosaccharides available to the world

12:10 Antoni Planas, University Ramon Llull, Spain
Mechanism and engineering of Bifidobacterium Lacto-N-biosidase for type I HMO synthesis

12:30 Stephan Warnke, Ecole Polytechnique Fédérale de Lausanne, France
IMS-IMS coupled to cryogenic IR spectroscopy for the identification of human milk oligosaccharides

12:50 Thomas Rexer, MPI DCTS – BPE Synthetic Biotechnology & eversyn, Germany
Activated sugars for oligosaccharide synthesis

13:10 Lunch

Session 4: Glycoengineering of Biopharmaceuticals

Chair: Mike Butler (NIBRT)

14:30 **Keynote Lecture:**
Horst Bierau, Merck Serono S.p.A., Rome, Italy
Glycosylation Challenges in Biopharmaceutical Manufacturing

15:10 David Falck, Leiden University Medical Center, Netherlands
PK beyond FcRn: How glycans impact pharmacokinetics of therapeutic antibodies

15:30 Marcelo Guerin, Biocruces Health Research Institute, Basque Country, Spain
Sculpting therapeutic monoclonal antibody N-glycans using endoglycosidases

15:55 Break with Coffee, Tea & Snacks

16:25 **Keynote Lecture:**
Lai-Xi Wang, University of Maryland, Maryland, USA
Antibody glycosylation and site-specific antibody-drug conjugates

17:05 Sebastian Malik, Roche Diagnostics, Switzerland
Implementation of in vitro glycoengineering of monoclonal antibodies into downstream processing

17:30 Christoph Gstöttner, Leiden University Medical Center, Netherlands
Multiplexed effector function assessment of antibody glycoforms using ACE-MS

17:50 **Lightning Talks** (5 x 5 min)
Ioscani Jimenez del Val, Ben West, Andrea Persson, Lars Stöckl, Anna-Barbara Hachmann

18:15 Poster Session and Dine & Discuss – Food, beer, wine and soft drinks are included

20:00 Einstein Lounge

THURSDAY, JANUARY 19TH, 2023

Session 5: Tools & Technologies for Glycoanalytics and Glycobioinformatics

Chair: Falk Büttner (Hannover Medical School)

9:00 **Keynote Lecture:**
Salome Pinho, University of Porto & i3S, Portugal
Glycans at the frontiers of chronic inflammation, autoimmunity and cancer: mechanisms and clinical implications

9:40 Mike Butler, NIBRT, Ireland
High-throughput glycosylation profiling applied to the analysis of Covid-19 virus evolution and changes in human serum following infection

10:05 Mario Schubert, University of Salzburg, Austria
Detection and quantification of α-Gal epitopes in intact monoclonal antibodies by NMR spectroscopy

10:25 Nathan Lewis, University of California, San Diego, USA
Correcting for sparsity and interdependence in glycomics to enhance discovery, diagnosis, and drug development

10:45 **Lightning Talks** (5 x 5 min)
Frania Zuniga-Banuelos, Friedrich Altmann, Yosra Helali, Michael Kudlich, Paras Kundalia

11:10 Break with Coffee, Tea & Snacks

11:40 Kelvin Anggara, Max Planck Institute for Solid State Research, Germany
Imaging Glycosylated Molecules One-At-A-Time

12:00 Leonhard Möckl, Max Planck Institute for the Science of Light, Germany
Biophysics, super-resolution microscopy, and bioengineering reveal structure-function relationships in glycocalyx biology

12:20 René Hennig, glyXera, Magdeburg, Germany
Clinical glycomics for the diagnosis of congenital disorders of glycosylation

12:45 Robert Woods, University of Georgia, Athens, USA
Glyfinder and glycoprotein builder: online tools for finding and modelling glycoproteins in the PDB

13:10 Closing remarks: Prof. Udo Reichl, Director, MPI Magdeburg

13:20 Lunch and End of the Conference

ABSTRACTS: TALKS

SESSION 1:
Glycosciences – Fundamentals and Advances in Clinical and Biopharmaceutical Research

Chair: Marcelo Guerin

Keynote Lecture:

Structure and mechanism of ER-luminal enzymes involved in protein glycosylation

Kaspar Locher

ETH Zurich, Zurich, Switzerland

Glycans of the SARS-CoV-2 Spike Protein

M Sanda[1,2,5], L Morrison[3], and R Goldman[1,4,5]

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The Covid-19 pandemic led to a worldwide health crisis, but also to the fastest development of vaccines and antiviral compounds to date, though a global collaboration. The basis for the development of effective therapeutics are detailed structural studies of the SARS-CoV-2 proteins. The SARS-CoV-2 spike glycoprotein mediates virion binding to the human cells through its interaction with the ACE2 cell surface receptors and is one of the prime immunization targets.
This study reports the analysis of the site-specific glycoforms with focus on the structural motifs of the identified O- and N- glycopeptides. To this end, high-resolution liquid chromatography–mass spectrometry (LC-MS/MS) as well as liquid chromatography-ion mobility spectroscopy-mass spectrometry (LC-IM-MS/MS) were used to study a recombinant SARS-CoV-2 spike full-length protein expressed in human embryonic kidney (HEK 293) cells.
The presented analyses identified 9 occupied O-glycosites and 17 N-glycosites. For the first time, LacdiNAc and polyLacNAc structural motifs associated with the N-glycopeptides were resolved. Novel O-glycopeptides including glycopeptides near the furin cleavage site (T678) of the spike glycoprotein were identified. Core1, core2 and extended core 1 type glycans with α-2,3 and α-2,6 sialic acid linkage were identified using cyclic ion mobility.
This study substantially expands the current knowledge of the spike protein's glycosylation and structure. In addition, it enables further functional studies, specifically the investigation of the impact of O-glycosylation on its proteolytic activation.

References

M. Sanda, L. Morrison, R. Goldman *(2021) Anal. Chem.* 2021, 93, 4, 2003–2009

Multiregional comparative N-glycomics reveals both spatial and phylogenetic gradients in mammalian brain N-glycome complexity

TS Klarić[1,2,†], I Gudelj[1,2,3,†], G Santpere[1,4], AMM Sousa[5,6], M Novokmet[2], F Vučković[2], S Ma[1], I Bečeheli[2], CC Sherwood[7], JJ Ely[7,8], PR Hof[9], D Josić[3,10], G Lauc[2,11], N Sestan[1,12]

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[†] These authors contributed equally to this work.

Numerous comparative “omics” studies have revealed unique aspects of human neurobiology, yet an evolutionary perspective of the N-glycome in mammalian brains is lacking. Here, we performed a detailed structural characterization of rat, rhesus macaque, chimpanzee, and human N-glycomes from four brain regions (the dorsolateral prefrontal cortex, hippocampus, striatum, and cerebellum) using chromatography combined with mass spectrometry. Despite the existence of a common template across all brain N-glycomes, we report distinct regional variation characterized by a gradient of increasing N-glycome complexity from the hindbrain to the cerebral cortex in all species. This trend is inversely correlated to the pattern of evolutionary conservation of brain regions in vertebrates. Namely, our data suggest that highly conserved brain regions that have retained their ancient configuration also have a higher proportion of ancient, minimally processed N-glycans, while brain regions that display a higher degree of evolutionary change across species have acquired N-glycoprofiles of greater complexity. Furthermore, we show that the evolution of the brain N-glycome in hominini has been characterized by a global increase in complexity coupled with greater usage of α(2-6)-linked N-acetylneuraminic acid. Finally, by integrating complementary donor-matched N-glycomic and transcriptomic data, we show that the primate brain N-glycome evolves at a faster rate than does the transcriptional framework underpinning it, providing a mechanism for rapidly generating additional diversity between closely related species. Our findings shed light on the evolutionary trajectory of the mammalian neuroglycome and suggest that human brain evolution, and the parallel evolution of higher cognitive processes, was accompanied by an increase in the diversity and complexity of sugar modifications found on N-glycoproteins.

Neolactotetraosylceramide is a novel biomarker for bladder cancer

IB Moreira[1], C Rossdam[1], J Beimdiek[1], J Schmitz[2], JH Braesen[2], FFR Buettner[1]

[1] Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany.
[2] Institute for Pathology, Nephropathology Unit, Hannover Medical School, Hannover, Germany.

Tumor cells display a set of modifications that confers them selective advantages. Particularly, alterations in glycosylation pathways are a common feature of all cancer hallmark abilities, with most FDA-approved tumor markers being glycan-based. Bladder cancer (BC) is a significant public health concern, being the tenth most common cancer worldwide. Currently, diagnosis of BC still relies on invasive methods, mainly cystoscopic examination. Regarding the non-invasive tests available, on the one hand, urine cytology is not reliable as a primary diagnostic tool, showing an overall sensitivity and specificity of up to 48% and 86%, respectively. On the other hand, the existing molecular biomarkers lack formal indication for clinical practice. Therefore, there is an urgent need to identify new biomarkers for the reliable detection of BC. Here we show that specific glycosphingolipid (GSL) structures are significantly increased in BC samples. We optimized multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF) to unravel for the first time the global GSL profile of formalin-fixed paraffin embedded primary bladder tumors and urine samples from BC patients. First, we found that GM3 ganglioside, globotriaosylceramide (Gb3) and neolactotetraosylceramide (nLc4) glycans are increased in BC when compared to the correspondent paired normal adjacent tissue. Immunofluorescence staining of tumor biopsies using antibodies against the three glycan structures confirmed their cancer-specific expression. Additionally, xCGE-LIF analysis of urine samples showed that nLc4 is increased in BC when compared to healthy controls, with an overall sensitivity of 57% and a specificity of 90%. To evaluate nLc4 realistic prospects of a clinical application, we did an enzyme linked immunosorbent assay (ELISA) and tested an equal number of urine samples from BC and healthy control individuals. This resulted in an overall sensitivity and specificity of 89% and 78%, respectively. Our current data incites the application of xCGE-LIF as a high-throughput compatible approach for the rapid and comprehensive screening of new potential biomarkers derived from different types of human biosamples. Moreover, our results demonstrate the power of nLc4 as a non-invasive BC urine detection marker, with the potential of improving its diagnosis.

References

Sung, H., et al. (2021) “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.” CA Cancer J Clin **71**(3):209-249.

Yafi, F.A., et al. (2015). “Prospective analysis of sensitivity and specificity of urine cytology and other urinary biomarkers for bladder cancer.” Urol Oncol **33**(66):e25-31.

Rossdam, C., et al. (2019). “Approach for Profiling of Glycosphingolipid Glycosylation by Multiplexed Capillary Gel Electrophoresis Coupled to Laser-Induced Fluorescence Detection To Identify Cell-Surface Markers of Human Pluripotent Stem Cells and Derived Cardiomyocytes.” Analytical Chemistry **91**(10):6413-6418

Fermentation-free and enzymes-based synthesis of vaccine antigens from *Neisseria meningitidis*, *Actinobacillus pleuropneumoniae* and *Haemophilus influenzae*

JO. Cifuente[1,2,#], J Schulze[3,#], A Bethe[3,#], V Di Domenico[1,#], C Litschko[3], I Budde[3], L Eidenberger[4], H Thiesler[3], I Ramón Roth[3], M Berger[3], H Claus[5], C D'Angelo[1,2], A Marina[2], R Gerardy-Schahn[3], M Schubert[6], ME. Guerin[1,2,7,], and T Fiebig[3]

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Capsular polysaccharides (CPS) are important virulence factors that protect bacterial pathogens from the host immune system. They are structurally diverse and can consist exclusively of saccharide units, or sugars alternating with phosphate or polyol-phosphate moieties. CPS can be used as antigens in highly effective glycoconjugate vaccines, in which they are coupled to a carrier protein to induce a T-cell dependent immune response. The manufacturing of glycoconjugate vaccines includes the purification of CPS from pathogen culture. To reduce biohazard and costs, chemical and enzymatic synthesis have been extensively studied as alternatives for CPS production.

Our research focusses on the biochemical and structural characterization of capsule biosynthesis enzymes and the development of enzyme-based synthesis cascades for the provision of CPS. The enzymes required for these protocols include (i) nucleotide-sugar epimerases and nucleotidyltransferases to generate substrates like nucleotide sugars and polyols, (ii) capsule polymerases using said substrates to polymerize the CPS, and (iii) CPS-modifying transferases that add glycosyl- or O-acetyl groups to e.g. distinguish between serotypes.

Here, we present the biochemical and mechanistic characterization as well as the biotechnological exploitation of capsule biosynthesis enzymes required for the generation and modification of phosphate-containing CPS from the Gram-negative pathogens *Neisseria meningitidis*[1], *Haemophilus influenzae*[2] and *Actinobacillus pleuropneumoniae*[3,4].

References

1. Fiebig T et al. (2020). Nat. Commun **11**:4723. https://doi.org/10.1038/s41467-020-18464-y
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3. Litschko C et al. (2021). MBio **12**:e0089721. https://doi.org/10.1128/mBio.00897-21
4. Litschko C et al. (2018). MBio **9**:16017. https://doi.org/10.1128/mBio.00641-18

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Differential *N*-Glycosylation Site Occupancy Depends on Distinct Amino Acid Sequence Features

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Abstract:
N-glycosylation is an essential co/post-translational modification of proteins in eukaryotes, which involves the transfer of glycan from lipid-linked oligosaccharide (LLO) to select asparagine side chains in Asn-Xaa-Thr/Ser (Xaa≠Pro) sequons of newly synthesized polypeptides in the lumen of the endoplasmic reticulum. *N*-glycosylation directly affects protein folding and plays important roles in protein function, stability, solubility, secretion, resistance to proteases and temperature, and enhancing half-life. Interestingly, the importance of *N*-glycosylation is highly variable between different glycosylation sites. However, the factors that determine the efficiency of site-specific *N*-glycosylation are not well understood due to the lack of tools to quantify site-specific glycosylation occupancy. Here, we aimed to understand how control of LLO biosynthesis affected site-specific *N*-glycosylation occupancy. We developed and optimized a targeted DIA LC-MS/MS MRM-HR method for quantifying site-specific occupancy at diverse *N*-glycosylation sequons in yeast cell wall glycoproteins and used this method to compare global site-specific glycosylation under two LLO stress conditions: deficiency of Alg6p or Alg7p, enzymes which catalyze distinct key steps in LLO biosynthesis. We found that a subset of *N*-glycosylation sites was differentially occupied in these different LLO stress conditions, consistent with active regulation of site-specific *N*-glycosylation depending on distinct amino acid sequence features surrounding the glycosylation sequons. Our results are consistent with a model in which cells under glycosylation stress maintain efficient glycosylation at critical sites in glycoproteins through regulated recognition of specific extended *N*-glycosylation sequons.

Rutinosidase: Glycosidase with unprecedented glycosylation activities controlled by a substrate tunnel

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Keywords: Glycosidase, transglycosylation, substrate tunnel, glycosyl azide.

Rutinosidases (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosidases, EC 3.2.1.168, CAZy GH5) cleave the glycosidic bond between the rutinose and the aglycone. Rutinosidase from *A. niger* also exhibits β-D-glucopyranosidase activity, and thus can be considered as a β-glucosidase with broad substrate specificity. This enzyme employs rutin as a rutinosyl donor or quercetin-3-β-glucoside as a glucopyranosyl donor. The crystal structure revealed strong and specific binding of aromatic aglycon (quercetin) and high tolerance at the glycon binding site. This enzyme can glycosylate (both with rutinosyl and β-glucosyl) many substrates such as alcohols (primary, secondary, and tertiary), phenols, and aromatic acids (glycoside at carboxy groups). Non-aromatic glycosylation products are no longer enzymatically hydrolyzed. Even the wild type rutinosidase can glycosylate azide to form β-rutinosyl or β-glucosyl azide, which is a yet undescribed phenomenon in the nonmutated glycosidases. The active-site mutant E319A can generate α-rutinosyl (or α-glucosyl) azide (Kotik et al, 2021). The unique abilities of this enzyme are plausibly caused by a substrate tunnel in the structure of rutinosidase, which may explain the unusual catalytic properties of this glycosidase and its strong and unusual transglycosylation potential (Brodsky et al, 2020).

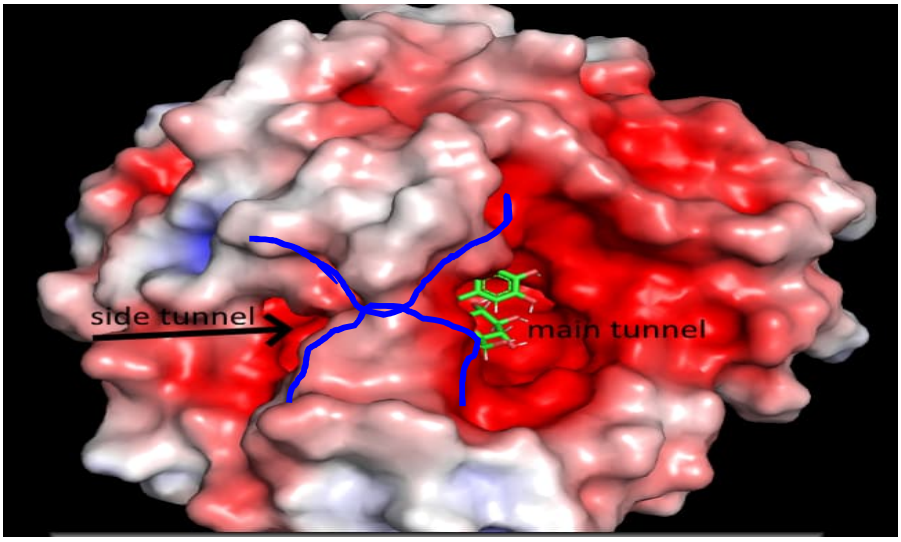

Figure 1. Substrate tunnels of rutinosidase from *A. niger* (rutin in the active site). The bridge over the active site building the side tunnel is formed by interactions of two loops (blue).

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Efficient thiodigalactoside-derived inhibitors of tandem-repeat galectins

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Human galectins are a family of twelve proteins that participate in a number of pathologies such as fibrosis, carcinogenesis, and metabolic disorders (Heine et al 2022). Their scavenging by β-galactoside-derived inhibitors is a promising therapeutic pathway (Laaf et al 2019). Although the biological roles of some members of this family, including the prototype Gal-1 and the chimera-type Gal-3, have been widely studied, the knowledge of the functions and ligand specificities of a structurally distinct group of tandem-repeat galectins are quite scarce. Tandem-repeat galectins feature two different carbohydrate-recognition domains interconnected with a peptide linker. We present here the synthesis of a library of 3-*O*-disubstituted thiodigalactoside-derived glycomimetics and their affinity to two tandem-repeat galectins, Gal-8 and Gal-9. The glycomimetics were synthesized in two reaction steps: dibutyltin oxide-catalyzed 3,3´-*O*-disubstitution of commercially available unprotected thiodigalactoside by propargyl bromide, followed by conjugation of various aryl substituents by copper-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC) (Vrbata et al 2022). The prepared glycomimetics were tested for inhibition of Gal-8 and Gal-9 by ELISA assay, and the affinities were compared to the established galectins Gal-1 and Gal-3. The introduction of C-3 substituents led to almost 50-fold increase in affinity compared with underivatized TDG. The structure-affinity relationships within the studied set were discussed using molecular modeling. In addition, the influence of multivalency was investigated for selected compounds. This is a pioneer study on the synthetic inhibitors especially of Gal-9; the identified lead compounds may be further used in biomedical research.

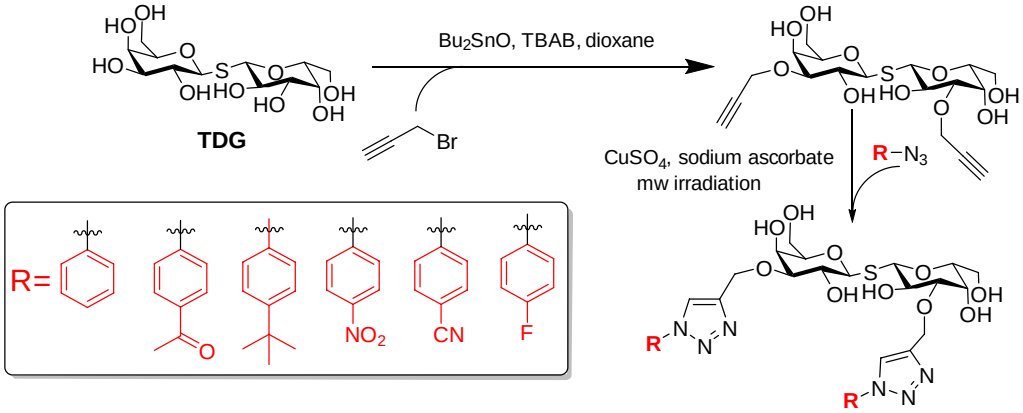

Figure 1. Scheme of the synthesis of glycomimetics for targeting of tandem-repeat galectins.

Support from the grant project 22-00262S by the Czech Science Foundation is gratefully acknowledged.

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Synthesis of synthetic lipid-linked oligosaccharides for in-vitro glycoengineering of hemagglutinin peptides by a cell-free, multi-enzyme cascade

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A wide range of peptides can be chemically synthesised or recombinantly expressed in bacteria in an aglycosylated form. To investigate the effect of *N*-glycosylation of these peptides regarding their various functions, i.e. protein stability and receptor binding, an efficient in-vitro *N*-glycosylation system is required.

Here, we present a cell-free system mimicking the Endoplasmic Reticulum glycosylation machinery of eukaryotes. This includes, in particular, the enzymatic transfer of glycans from a lipid-linked (LL) precursor via an oligosaccharyltransferase (OST) onto a peptide. In a first step, a one-pot multi-enzyme cascade employing a set of recombinant glycosyltransferases was established to generate lipid-linked oligosaccharides (LLO) as glycosylation substrates. Using pythanol as a lipid anchor, core-mannose as well as novel hybrid and complex LLOs were generated successfully. In a second step, the single-subunit OST STT3A from *Trypanosoma brucei* was used to transfer these unpurified substrates to the consensus sequence (Asn-X-Ser/Thr) of a synthetic peptide. It is demonstrated that STT3A also shows activity towards simple hybrid structures such as LL-GlcNAc2Man3GlcNAc1Gal1 or the simple complex core-structure LL-GlcNAc2Man3GlcNAc2. Finally, to demonstrate the successful application of the platform to modify polypeptides, we *N*-glycosylated influenza A virus hemagglutinin peptides (HA1) (strain: A/Puerto Rico/8/1934) that were recombinantly expressed as aglycosylated form in *E. coli*. Subsequently, these HA1 peptides were modified using several glycosyltransferases to generate galactosylated and sialylated glycans.

In summary, cell-free in-vitro enzyme cascades were established to synthesize LLOs, i.e. LL-GlcNAc2Man3GlcNAc1Gal1 and LL-GlcNAc2Man3GlcNAc2. As an example for application, we in-vitro *N*-glycosylated HA1 peptides to generate complex galactosylated and sialiylated glycans. In the future, the toolbox established will be applied to glycosylate biomedical important peptides with desired glycans to investigate the impact of defined glycosylation on protein function. In addition, we are exploring options for the use of our platform to produce protein-based vaccines.

Association between polygenic risk scores for plasma protein N glycosylation traits and 276 ICD-10 diseases

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N-glycosylation influences the physical and biological properties of proteins and is involved in maintenance of tissue structure, cell signaling, immune response, etc. The human blood plasma N-glycoprofile is altered in disease, thus being a promising molecular marker of health status. The connection between N-glycosylation and pathologies could be partially attributed to the shared genetic architecture of these traits. The aim of this study is to investigate the association and possible causal relationships between 117 human plasma N-glycosylation traits and 276 diseases included in the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) list. Based on previously conducted genome wide association studies (GWAS) of plasma N-glycome we developed polygenic risk score (PRS) models for 117 N-glycan traits and tested their association with ICD-10 diseases in the UKBB cohorts. We have identified 35 plasma N-glycosylation traits to be statistically significantly associated with at least one ICD-10 phenotype and 18 ICD-10 phenotypes to statistically significantly associate with PRS for a least one N-glycosylation trait. A half of such ICD-10 phenotypes were related to the health of cardiovascular system. The abundance of the high-mannose structure M9 was often associated with cardiovascular phenotypes. For the 71 pairs of statistically significantly associated glycan traits and diseases we performed bidirectional Mendelian Randomization analysis to investigate the causal relationships between these traits using GWAS summary statistics for these traits available in-house or from UKBB studies. We found evidence that a) abundance of oligomannose structures in total plasma glycans may have a causal role in the onset of hypertension, and b) disorders of lipoprotein metabolism may cause increased levels of the oligomannose glycan M9 in the plasma N-glycome. Found causal relationships provide basis for further development of glycome-based drug targets and biomarkers.

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Lacto-*N*-tetraosyl ceramide is a novel marker for human pluripotent stem cells

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Human pluripotent stem cell-derived somatic cells are envisioned to be applied in regenerative medicine in future. Thereby, the differentiated cells cannot contain any residual pluripotent stem cells, which might otherwise lead to teratoma formation in the human recipient by uncontrolled proliferation. The glycosphingolipid-based pluripotency markers SSEA3, SSEA4, and SSEA5 are commonly applied to characterize the pluripotent state of stem cells and can also be used for elimination of residual pluripotent stem cells from differentiated populations. Applying our novel analytical approach for profiling of glycosphingolipid glycosylation by multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF) [Rossdam *et al.*], we set out to reassess human pluripotent stem cell-specific glycosphingolipids. Thereby, we observed that levels of the glycosphingolipid-derived glycan lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Lc4) were considerably reduced upon differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) into early stages of the three germ layers mesoderm, endoderm, and ectoderm, suggesting the potential of Lc4 as a novel pluripotency marker. The decrease of Lc4 levels during the first four days of differentiation was confirmed by flow cytometry upon live cell surface staining using an Lc4-specific antibody. Using this antibody, we could additionally sort the cells into an Lc4-positive and Lc4-negative population by magnetic cell separation (MACS). We were able to show by flow cytometry that the Lc4-negative cells are still positive for SSEA3, SSEA4, and SSEA5 while qPCR-based analysis of relative expression levels of additional stem cells markers, *OCT4* and *NANOG*, already revealed a differentiated state. In contrast, expression of *OCT4* and *NANOG* was much higher in the Lc4-positive cell population. These findings validate Lc4 as a novel and promising marker for undifferentiated stem cells, even suggesting that this marker is more specific for pluripotency than SSEA3, SSEA4, and SSEA5.

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Why are Cys domains of mucins not C-mannosylated?

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Abstract:

Mucins are the major component of the mucus, the first biological barrier at the surface of secretory epithelia. Mucins are high molecular weight glycoproteins and they occur secreted or membrane-bound. Serine-, threonine- and proline-rich regions are highly O-glycosylated. These S/T/P regions are interrupted by Cys domains (CysDs), which are small domains with 8 conserved cysteine residues. CysDs contain conserved WxxW motifs, the consensus for C-mannosylation. Therefore, it has been hypothesized that CysDs are C-mannosylated. However, previous studies could not proof this. Hence, we investigated the potential C-mannosylation on mucin CysDs by mass spectrometry. We recombinantly expressed CysD5 of human MUC5AC in CHO cells. MUC5AC is a secreted, gel-forming mucin. We purified His-tagged CysD5 with Ni-NTA-chromatography and analyzed digested peptides by LC-MS. MS analysis revealed that this CysD is not C-mannosylated, even if the protein is retained to the ER.
However, we showed that CysD are C-mannosylated if conserved cysteine-residues are mutated. The mutated CysDs were almost fully C-mannosylated. These cysteine-residues form intramolecular disulfide bridges. We compared these results to the CysD from cartilage intermediate layer protein 1 (CILP1). This CysD contains the conserved WxxW motif but not the mentioned cysteine-residues. In CHO cells, CILP1 CysD is C-mannosylated.
Our experiments suggest that the motif of mucin CysDs is a potential substrate for C-mannosyltransferases, but cysteine-residues hinder C-mannosylation. We propose that formation of intramolecular disulfide bridges or co-translational binding of chaperones to adjacent cysteine-residues prevents C-mannosylation.

Sialic acid decorated-allergens suppress inflammation

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House Dust Mite (HDM) allergy is an inflammatory respiratory disease affecting more than 50 million people worldwide and is one of the most important risk factors for the development of asthma. Available allergen immunotherapy, consisting of administering incremental doses of the allergen from HDM over a period of 3-5 years to induce tolerance, is lengthy and carries a high risk of severe side effects. New therapeutic options that are safer and can rapidly induce tolerance are therefore warranted. One of such therapeutic options includes the use of carbohydrate-based protein conjugates to target immunomodulatory receptors on the immune cells that are involved in the development of allergy. Sialic acid–binding immunoglobulin-like lectin-9 (Siglec-9) is an immunomodulatory receptor found predominantly on immune cells. It is an important negative regulator of acute inflammatory responses and is a potential target for the treatment of HDM allergy. We describe a Siglec-targeting platform consisting of an allergen of HDM, *Dermatophagoides pteronyssinus (Der p 2*), decorated with a natural Siglec-9 ligand, (α2→3) N-acetylneuraminic acid (sia-*Der p 2*). Treatment of human peripheral blood mononuclear cells (PBMCs) with these glycoconjugates blocked the production of anti-CD3/CD28-induced allergy-associated inflammatory cytokines and augmented the expression of anti-inflammatory cytokine, interleukin-10 (IL-10). The glycoconjugates also suppressed the activation of CD4 T helper cells. Moreover, we also established that sia-*Der p 2* expanded a population of T regulatory cells known to suppress the function of pathologic CD4 T helper cells. Collectively, these results demonstrate a promising potential of targeting Siglec receptors with glycan-based constructs for the rapid induction of an anti-inflammatory state in immune cells for short-lasting allergen immunotherapy for HDM.

Polysaccharides as Tumor Therapeutics, 1868-2023

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Busch (1866,1868) and Fehleisen (1880) discovered sarcoma patients with nosocomial or induced ***erysipelas*** skin infections had tumor regression or elimination. Spronck (1891) showed that heat treated "*Streptococcus erysipelatos*" (now *S. pyogenes*) cultures, injected into humans or dogs with sarcoma caused tumor regression. Coley (1891, 1910) used a mixture of bacteria-filtered, heat treated *S. erysipelatos* and *Bacillus prodigiosus (Serratia marsescens)* culture media to treat sarcoma patients. Heat stability today suggests polysaccharides were the active principle. (API). "Coley's Toxin" was produced by Parke-Davis for 20 years with widely variable potency. Reviews on Coley's Toxin by Nauts, *et al*., 1990, and Novotny (1985) showed 5 year survival times of nearly 50% in 897 patients with 18 different cancers. Shear, *et al.* investigated Serratia API, in 1943, Hartwell and Shear isolating a *Serratia* polysaccharide (we call PS1), lacking peptide, containing lipid, phosphate, that caused tumor-specific hemorrhage in mice. PS1 was 1500 times more potent than Parke-Davis' Coley's Toxin. Algire, 1947, microscopically showed PS1 caused tumor-specific capillary damage in mice. *Repeating Shear's work in our lab, PS1 was a 250,000da polysaccharide by SEC and HPLC, which caused tumor hemorrhage in a new mouse model.* Hellerqvist, et al. at Vanderbilt,1980's isolated a 270kDa glycan CM101 (GBS toxin) from *Streptococcus agalactica* with lipid and phosphate attached which caused lung capillary damage, 50% fatality in human Early Onset Disease. CM101 also caused tumor specific capillary damage, hemorrhage in rodents. Phase I clinical trials showed 33% effectivity in stage 4 patients. (DeVore, et al. 1997). *The capillary endothelial GBS toxin receptor Sialin (SLC17A5) was proposed by Fu, et al. in 2002, which we have expression cloned in HeLa cells.* Mechanism was complement activation, inflammatory cytokine cascade and tumor specific neutrophil capillary destruction. Voelz, et al., 2010, reported nosocomial Serratia in a neonate facility. In the first 5 days 9 neonates had respiratory distress, febrility, 50% fatality similar to the GBS effects on newborns. Thus, capillary endothelium receptors for CM101 and Serratia PS1 may be identical. *We believe polysaccharides CM101 and PS1 are the API's of Coley's Toxin.*

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SESSION 2:
Novel Emerging & Enabling Systems in Glycobiotechnology

Chair: Mattias Collin

Keynote Lecture:

Automated glycan assembly as basis for life science and material science applications

Peter Seeberger

Max Planck Institute of Colloids and Interfaces, Germany

Generation of glycan-specific nanobodies

SK Khilji,[1,2,7] F Goerdeler,[1,2,7] K Frensemeier,[1,2] D Warschkau,[1,2] J Lühle,[1,2] Z Fandi,[1] F Schirmeister,[1] ZA Chen,[3] O Turak,[4] A Mallagaray,[4] S Boerno,[5] B Timmermann,[5] J Rappsilber,[3,6] PH Seeberger,[1,2,7*] and O Moscovitz[1,7,*]

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The development of antibodies that target specific glycan structures on cancer cells or human pathogens poses a significant challenge due to the immense complexity of naturally occurring glycans. Automated glycan assembly enables the production of structurally homogeneous glycans in amounts that are difficult to derive from natural sources. Nanobodies (Nbs) are the smallest antigen-binding domains of heavy chain-only antibodies (hcAbs) found in camelids. To date, the development of glycan-specific Nbs using synthetic glycans has not been reported. By using defined synthetic glycans for alpaca immunization we prove the formation of glycan-specific hcAbs. Next, we identified, isolated, and produced Nb specific for the tumor-associated carbohydrate antigen Globo-H. The Nb binds the terminal fucose of Globo-H and recognizes synthetic Globo-H in solution and native Globo-H on breast cancer cells with high specificity. These results demonstrate the potential of our approach for generating glycan-targeting Nbs to be used in biomedical and biotechnological applications.

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Towards Automated Enzymatic Glycan Synthesis with Microgel Immobilized Glycosyltransferases in a Countercurrent Flow Reactor

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Enzyme-assisted glycan synthesis is an attractive alternative to chemical synthesis as it has the advantage of achieving quantitative regio- and stereoselective glycosylation in a single step. In addition, automated glycan synthesis with immobilized glycosyltransferases offers several advantages including cascading, handling, and re-use of the biocatalysts as well as facile product isolation. Although several strategies for glycosyltransferase immobilization have been published, only a few a have been applied for automated glycan synthesis (Heinzler et al. 2019; Wen et al. 2018).

Here we want to give insight in published automated enzymatic glycan synthesis approaches and present a novel strategy for a **mi**crogel countercurrent flow **r**eactor for **a**utomated **g**lycan synthesis with immobilized **e**nzymes (**MiRAGE**). With MiRAGE, we address multiple issues in recent enzymatic glycan synthesis processes. Important steps towards the accomplishment of automated enzymatic glycan synthesis include: (i) immobilization of enzymes in functional and biocompatible microgels; (ii) design and construction of a membrane-based countercurrent flow reactor with automated product purification; (iii) in silico simulation of immobilized enzyme cascades for optimization of bio-processing; (iv) development of a fast at-line glycan analysis platform. Our envisaged solutions include scalable concepts for the immobilization of enzymes with compartmentation of enzymatic cascades for the production of glycans and simultaneous, continuous removal of by-products, combined in an automated, countercurrent flow reactor.

Recent achieved aims focussed on production and characterization of several glycosyltransferases for application in enzymatic cascades. Furthermore, immobilization of glycosyltransferases into microgels was improved towards embedment into a countercurrent flow reactor. With economic gram scale synthesis of nucleotide sugars automated glycan becomes even more feasible (Fischöder et al. 2019; Frohnmeyer et al. 2022; Rexer et al. 2021).

To this end, our project “MiRAGE will lay important advancements in automated enzymatic glycan synthesis by developing a novel biohybrid platform technology using microgel-immobilized enzyme cascades as reaction compartments and integrating them into an automated countercurrent compartment flow reactor.

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SUGAR-TARGET: An immobilised enzyme cascade for targeted and bespoke glycosylation

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N-linked glycosylation is a critical process for biotherapeutics as it can improve drug efficiency, efficacy, and half-life. It is therefore desirable to control N-linked glycosylation, to generate bespoke drugs with enhanced activity. However, this can be a challenging task since glycosylation is a non-templated and complex process owing firstly to the promiscuity of the enzymes involved and secondly to enzyme and nucleotide sugar donor availability. This also results in heterogeneity amongst cell-derived glycoproteins, limiting therapeutic efficacy. Despite the intense efforts to control glycosylation, current methods face important limitations including complexity and lack of homogeneity. Here, we present a novel platform comprising sequential glycosylation reactions for tailored sugar structures (SUGAR-TARGET) using immobilised enzymes, allowing controlled and bespoke N-linked glycosylation of glycoproteins in a cell-free environment (Makrydaki et al, 2022). Specifically, this novel system consists of immobilised glycosyltransferases (GnTI, GalT and SiaTs) and glycosidase enzymes (ManII), that comprise a glycosylation pathway where promiscuity naturally exists. We designed and applied an *in vivo* biotinylation method that expresses glycosyltransferases *in E. Coli* and purifies/immobilises them in one-step. The immobilised enzymes were used in a sequential fashion to reconstruct an N-linked glycosylation pathway on free glycans and on a panel of proteins produced in mammalian and glycoengineered microbial hosts. SUGAR-TARGET resulted in increased glycoform homogeneity (>95% conversion). Finally, immobilised GalT was used to enhance the galactosylation profile of three IgGs, yielding 80.2 – 96.3 % terminal galactosylation. Enzyme recycling was further demonstrated for 7 cycles, with a combined reaction time greater than 140 hours. The methods and results outlined in this work demonstrate the application of SUGAR-TARGET as a protein-independent *in vitro* glycosylation strategy applied post-expression that is easy to implement, scalable, modular and reusable.

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Makrydaki, E. *et al.* Immobilised Enzyme Cascade for Targeted Glycosylation. *bioRxiv* 2022.04.08.487553 (2022) doi:10.1101/2022.04.08.487553.

Parallel synthesis of glycans by vapor coupling

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The investigation of molecular interactions between proteins and oligosaccharides is of great interest for the discovery of new drug candidates, diagnostics, and vaccines. This typically requires access to large glycan libraries. Compared to the well-established parallel peptide and oligonucleotide synthesis methods, chemical glycosylation typically requires inert and temperature-controlled conditions. Thus, the parallel chemical synthesis of oligosaccharides remains a major challenge.

To enable this, we adopted the SPOT synthesis on cellulose membranes by Frank et al.[1] and devised a novel vapor-based strategy[2], to ensure controlled conditions suitable for glycosylation reactions. First, building blocks are spotted at room temperature onto cellulose membranes, which are functionalized with a base-labile linker. Then, the spotted membranes are transferred to a custom-built instrument and cooled to –15°C. Activation of the glycosyl donor is achieved by delivery and condensation of activator and solvent vapor inside the glycosylation chamber. After the reaction, the membranes are washed and the temporary protecting groups are removed for the next synthesis cycle. These steps are repeated until the target structures are formed in parallel. Deprotection of the ester protecting groups and release of the products from the surface is achieved by a base, followed by purification and characterization.

With this proof-of-concept VaporSPOT approach, we showed the parallel synthesis of six different oligosaccharides and up to four residues in length in the micromolar scale (~1 µmol). The method offers a flexible and cost-efficient way to rapidly screen the glycosylation outcome of different glycosyl donors in parallel and synthesize oligosaccharides in good purity on micromolar scale.

Currently, we apply our laser-based technology[3-6] to perform an on-chip synthesis of glycopeptides and glycans, directly in the microarray format. We can deposit tiny amounts of different building blocks embedded in an inert polymer matrix onto a functionalized glass slide by using our laser system. Then, these patterns can be reacted in a chemical vapor, retaining the spot pattern. This will allow us to chemically synthesize glycans in parallel on surfaces and screen for e.g. lectin binding.

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Enzyme cascades for the synthesis of nucleotide sugars: Updates to recent production strategies

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Nucleotide sugars play an essential role in nature and serve as natural substrates for Leloir glycosyltransferases. However, effective production systems for nucleotide sugars are spare or poorly discussed. We discuss developing new approaches to nucleotide sugar synthesis and production strategies based on critical production parameters such as the space-time yield (STY), the total turnover number (TTN), and product yields. Valuable nucleotide sugars such as UDP-Gal, UDP-GalNAc, and UDP-GlcNAc were produced in the multi-gram scale by applying the repetitive-batch (rep-batch) procedure (Fischöder et al. 2019). Extraordinarily high TTNs of 494 g_P/g_E (UDP-Gal), 522 g_P/g_E (UDP-GlcNAc), and 398 g_P/g_E (UDP-GalNAc) were obtained for a 5-day production week (Fischöder et al., 2019). Economic access to UDP-GlcA, UDP-GlcNAc, and GDP-Fuc was developed by integrating an ATP regeneration system with polyphosphate kinase (PPK) and polyphosphate (polyP), leading to high conversion yields (Gottschalk et al. 2021 and Frohnmeyer et al. 2022). We demonstrated the synthesis of up to 1.6 g of GDP-Fuc in rep-batch mode with a final TTN of 27 g_P/g_E. However, a higher TTN of 31 g_P/g_E was reached with ATP excess. The latter synthetic approach converted up to 25% of the substrate to ADP-Fuc as site product. GDP-Fuc was purified by sequential steps of phosphate precipitation, where the nucleotide sugar was treated with an alkaline phosphatase solution in the first step, and >95% of orthophosphates were precipitated at -20 °C by supplementing the solution with 1.5 Volumes of 2-propanol and GDP-Fuc afterward precipitated by supplementing the solution by adding 2 volumes of 2-propanol. This protocol recovered 77% of GDP-Fuc (Frohnmeyer et al. 2022).
UDP-GlcA and UDP-GlcNAc were efficiently synthesized by enzyme cascade immobilization on magnetic beads (Gottschalk et al., 2022). Multiple uses of the magnetic beads up to the fifth cycle resulted in high average STYs of 4.3 $g*L^{-1}*h^{-1}$ for UDP-GlcA and 6.3 $g*L^{-1}*h^{-1}$ for UDP-GlcNAc (Gottschalk et al. 2022).

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Development of a cell-free enzymatic cascade for the synthesis of GDP-fucose - modeling and optimization.

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Glycans fulfill essential biological roles central to human health. They can be found in their free form or conjugated to proteins and lipids. To increase their availability for both scientific and commercial use, there is growing demand to develop economical synthesis routes. The nucleotide sugar GDP-fucose is the substrate for the biosynthesis of fucosylated glycans. However, GDP-fucose is scarcely available, at prices exceeding 120 €/mg as its production through approaches like fermentation or chemical synthesis is expensive and elaborate.

As an alternative, we have established a scalable, cell-free enzymatic cascade reaction to synthetize GDP-fucose from inexpensive precursors, polyphosphate GMP and fucose (Fig. 1). The initial design of reactions and conditions was based on previous work by Mahour et al. To optimize the final product titer and to minimize the biocatalyst load enough to obtain a substrate conversion of at least 90%, a model based on Michaelis-Menten kinetics was established. The model was built using a set of reaction data for parameter estimation; the software COPASI was used as a platform for all simulation and optimization steps. Using this approach, the product titer could be increased from the initial value of 16 mM to 19.8 mM after 24 hours. In addition, the biocatalyst load was decreased from 0.04 mmol/L to 0.02 mmol/L with substrate conversion close to 99%.

Overall, this work demonstrates the potential of rationally designed modeling and optimization approaches to improve cell-free synthesis methods. This is paving the way for the commercial use of the technology. Through our spin-off eversyn® (www.eversyn.de), we are planning to provide low-cost nucleotide sugars and glycans for applications in the (bio-) pharma and nutrition industries in the near future.

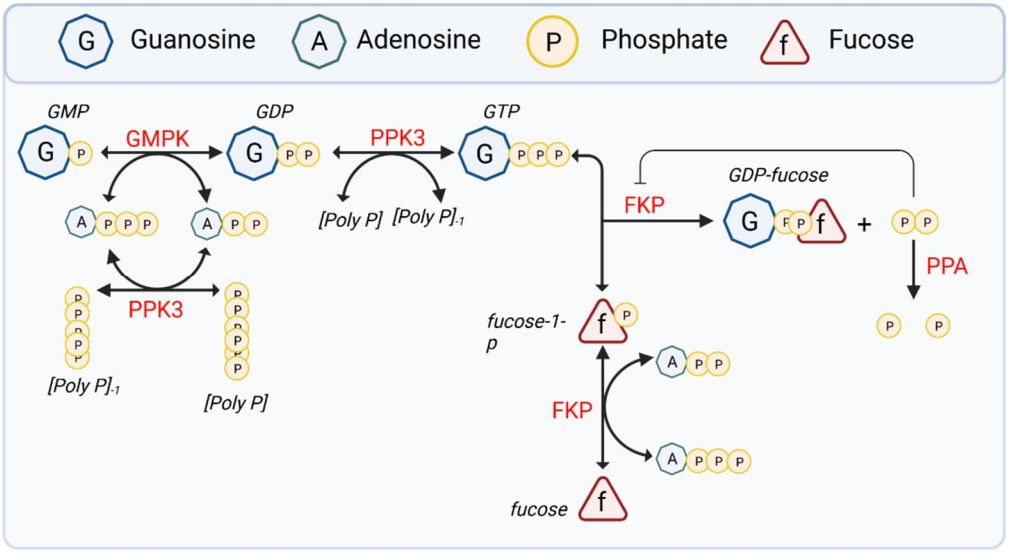

Figure 1 - Multi-enzyme cascade for the synthesis of GDP-fucose from fucose, polyphosphate and GMP.

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Development of an *in-vitro* multi-enzyme cascade for the synthesis of uridine diphosphate *N*-acetylgalactosamine using a DoE approach

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Biocatalytic production of active pharmaceutical ingredients, nutritional components and other valuable molecules is becoming increasingly important. In general, either cell-free or whole cell synthesis can be utilized. Both approaches exhibit several advantages and disadvantages (Tao et al., 2011). For instance, whole cell biocatalysts provide inherent co-factor regeneration, whereas in cell-free system undesired side reactions can be avoided (Straathof et al., 2000). Here, a cell-free multi-enzyme cascade producing the nucleotide sugar uridine diphosphate *N*-acetylgalactosamine (UDP-GalNAc) was developed. This activated molecule is an essential element of various glycoproteins and glycolipids in human milk that modulate the immune response of infants (Figueroa-Lozano et al., 2019). To date UDP-GalNAc is commercially only available in mg quantities with prices exceeding 70 €/mg.

The cascade established consists of six recombinant enzymes overexpressed in *E. coli* (Mahour et al., 2018; 2022). In one-pot batch reactions, the inexpensive substrates uridine, polyphosphate and GalNAc are converted to UDP-GalNAc. To reduce costs, ATP is *in situ* regenerated from polyphosphate. Starting at 30 °C, pH 7.5 and 45 mM $MgCl_2$ and a batch time of 24 h, a conversion yield of approximately 10 % and a final product titer of 2.9 g/L could be obtained from initial substrate concentrations of 50 mM uridine and GalNAc, respectively.

By employing a statistical design of experiments (DoE) approach, we aimed at increasing the product yield through optimization of the process parameters temperature, pH and $MgCl_2$ concentration. Therefore, an experimental range of 30-40 °C, pH 7-9 and 45-100 mM $MgCl_2$ was screened. By using a D-optimal design, a set of 21 experiments was conducted. The optimal conditions identified were 39 °C, a pH of 8.9 and 96.5 mM $MgCl_2$. Using these parameters, the yield could be increased to 80 % with a final UDP-GalNAc concentration of 26.8 g/L.

Through our spin-off eversyn® (www.eversyn.de) we will provide nucleotide sugars and glycans in large scales for applications in the (bio-)pharma and the nutrition industry in the near future.

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ALiCE®: High-yielding, scalable, eukaryotic cell-free protein expression for rapid production of N-glycoproteins

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Eukaryotic cell-free protein synthesis (CFPS) systems have the potential to simplify and speed up the expression and high-throughput analysis of complex proteins with functionally relevant post-translational modifications (PTMs). However, low yields and the inability to scale such systems have so far prevented their widespread adoption in protein research and manufacturing. Here, we present a detailed demonstration for the capabilities of ALiCE®, a CFPS system derived from *Nicotiana tabacum* BY-2 cell culture. Owing to this eukaryotic origin, ALiCE retains native organelle machinery in the form of 'microsomes', hybrid reformations of endoplasmic reticulum and Golgi components. The system is therefore able to express diverse, functional proteins at high yields in 48 hours, complete with disulfide bonds and N-glycosylation.

Recent advances in the scaling of BYL production methodologies have allowed scaling of the CFPS reaction and we show simple, linear scale-up of batch mode reporter protein expression from a 100 µL microtiter plate format to 10 mL and 100 mL volumes in standard Erlenmeyer flasks. Scaling of reactions yields greater amounts of protein products for more detailed characterisation. Thus, we present N-glycan analyses from a panel of interesting model proteins, specifically: a dimeric enzyme, glucose oxidase; the monoclonal antibody adalimumab and the SARS-Cov-2 receptor-binding domain. Functional binding and activity are shown using a combination of surface plasmon resonance and a serology-based ELISA method. Finally, in-depth PTM characterisation of purified proteins revealed the correct formation of intra- and intermolecular disulfide bonds, with homogenous, high occupancy N-glycan profiles typical of plant-derived glycoproteins i.e. high mannose structures with some plant-specific α1,3-fucosylation and β1,2-xylosylation.

Taken together, BYL provides a real opportunity for screening of complex proteins at the microscale with subsequent amplification to manufacturing-ready levels using off-the-shelf protocols. This end-to-end platform suggests the potential to significantly reduce cost and the time-to-market for high value proteins and biologics.

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Protective capacity of a recombinant vaccine against the cattle parasite *Ostertagia ostertagi* relies on engineering its native N-glycan composition

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With the rising resistance against anthelmintic drugs, a more sustainable control strategy against parasitic nematode infections in livestock, such as vaccination, is required. Vaccination with native activation-associated protein 1 from the economically important cattle parasite *Ostertagia ostertagi* (Oo-ASP-1) have demonstrated to induce protection (Meyvis et al 2007). However, collecting large quantities of Oo-ASP-1 is difficult as it requires inoculation and sacrifice of large numbers of animals, which is labour-intensive, time-consuming and ultimately unsustainable for large-scale production. For this reason, vaccine development is dependent on recombinant expression of this protein. Attempts have been made to express Oo-ASP-1 in, for instance, yeast and baculovirus expression systems, but failed to confer the same level of protective capacity as native Oo-ASP1 due to difference in protein folding and post-translational modifications (Geldhof et al 2008; González-Hernández et al 2016). Here, we show that glyco-engineering native glycan structures on recombinant Oo-ASP-1 expressed in *Nicotiana benthamiana* restores the protective capacity of the vaccine. Recombinant Oo-ASP-1 is easily expressed in high quantities in *N. benthamiana* and subsequently purified without complex downstream processing. Furthermore, the N-glycosylation of native Oo-ASP-1 is mimicked by co-expression of specific glycosyltransferases. In vaccination trials, calves vaccinated with recombinant Oo-ASP-1 demonstrated a strong reduction in faecal egg counts and a significant increase in local IgG1 and IgG2 antibody responses, which correlates with protective immunity. These results indicate that mimicking the native N-glycan composition of Oo-ASP-1 in *N. benthamiana* is required for its vaccine efficacy. Furthermore, these results demonstrate that N-glycosylation is an essential component for developing effective recombinant vaccines against parasitic nematodes.

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Glyco-gold nanoparticles as a targeting strategy for immune cells

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Glycans are the main key players to immunity. Specific glycosylation sites on immune cell proteins enhance migration throughout the body, being mimicked by pathogens or metastatic cancer cells, highlighting the biological importance of glycosylation for physiological and pathophysio-logical conditions. In order to mimic nature, the role of glycosylated gold nanoparticles (glyco-GNPs) as specific modulators of immune cells interactions was studied. First, one of the most abundant glycans expressed on the cells membranes, A2G2S2, which is a sialic acid-terminal gly-copeptide, was attached to the GNPs surface in order to avoid the tropism of NPs towards organs from the reticuloendothelial system (RES). The presence of the glycopeptide reduced liver up-take in healthy immunocompetent mice and helped to escape hepatic degradation. Second, su-praphysiological levels of mannose (Man) or sialic acid (Sia) can stimulate regulatory T cells dif-ferentiation, causing immunosuppression or tumor regression. This can be translated to autoim-mune diseases such as primary biliary cholangitis (PBC), which its only treatment is liver trans-plantation. In an animal model of PBC, a single dose of Man-GNPs was able to repolarize mac-rophages towards a restorative phenotype for more than 24 hours by impairing TNF-α, IL-1β, Arg1 and IL-6 production, making them an effective targeting strategy for selective autoimmune cells. Our study is a proof-of-concept that glycans are able to regulate immune cells responses, offering new insights on the role of glycans as immunosuppressors and potential of the emerging field of glycomedicine.

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O-Linked Sialoglycans Modulate the Proteolysis of SARS-CoV-2 Spike and Likely Contribute to the Mutational Trajectory in Variants of Concern

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The emergence of a polybasic cleavage motif for the protease furin in the SARS-CoV-2 spike protein has been established as a major factor for enhanced viral transmission in humans.[1–4] The peptide region N-terminal to that motif is extensively mutated in major variants of concern (VOCs) including Alpha, Delta and Omicron.[5–14] Besides furin, spike proteins from these variants appear to rely on other proteases for maturation, including TMPRSS2 that may share the same cleavage motif.[15,16] Glycans found near the cleavage site have raised questions about proteolytic processing and the consequences of variant-borne mutations.[17] With a suite of chemical tools, we identify that sialic acid-containing O-linked glycan structures on Thr678 of the SARS-CoV-2 spike influence cleavage by the host proteases furin and TMPRSS2 and posit O-linked glycosylation as a likely driving force for the emergence of mutations in VOCs. We provide direct evidence that the glycosyltransferase GalNAc-T1 primes glycosylation at Thr678 in the living cell, and this glycosylation event is suppressed by many, but not all variant mutations. A novel strategy for rapid bioorthogonal modification of Thr678-containing glycopeptides revealed that introduction of a negative charge completely abrogates furin activity. In a panel of synthetic glycopeptides containing elaborated O-glycans, we found that the sole incorporation of *N*-acetylgalactosamine did not substantially impact furin activity, but the presence of sialic acid in elaborated O-glycans reduced furin rate by up to 65%. Similarly, O-glycosylation with a sialylated trisaccharide had a negative impact on spike cleavage by TMPRSS2. With a chemistry-centered approach, we firmly establish O-glycosylation as a major determinant of spike maturation and propose that a disruption of O-GalNAc glycosylation is a substantial driving force for the evolution of VOCs.

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SESSION 3:
Synthesis and Function of Human Milk Oligosaccharides

Chair: Lothar Elling

Keynote Lecture:

Human milk oligosaccharides: New infant formula ingredients with diverse health benefits

Katja Parschat

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Simulated symbioses: how human milk oligosaccharides influence the nascent gut microflora

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The development of a stable human gut microbiota occurs within the first year of life, and there remain open questions about how microfloral species are influenced by the composition of human milk oligosaccharides (HMOs). We previously explored the space of possible HMO structures by modelling their synthesis [McDonald, A.G., et al. (2020)] and now investigate the effect of the HMO glycome on bacterial symbiosis and competition, based on the glycohydrolase (GH) enzyme activities known to be present in microbial species. In the initial phase of the work, we modelled the possible biosynthetic pathways of HMOs using a subset of 11 enzyme activities that accounted for 206 of 226 distinct HMOs isolated from human milk, via simulation performed within the Glycologue platform. Comparison of monosaccharide compositions demonstrated that the model was able to discriminate between two possible groups of intermediates between major subnetworks, and to assign possible structures to several previously uncharacterised HMOs. In the second phase of the work, we extracted from UniProt a list of all bacterial species catalysing glycohydrolase activities (EC 3.2.1.-), cross-referencing with the BRENDA database to obtain a set of taxonomic lineages and CAZy family data. A set of 13 GH enzyme activities was selected based on known activities to build our model. A diverse population of experimentally observed HMOs was then fed to the Glycologue simulator, and the enzymes matching specific bacterial species were recorded, based on individual enzyme occurrence in the UniProt dataset. Bacterial species sharing similar GH profiles were considered likely to compete for the same set of dietary HMOs within the gut of the newborn. Conversely, potential symbioses can be inferred from bacterial species that possess complementary enzyme profiles enabling the digestion of the HMO glycome. Both human GTs and putative bacterial GHs catalysing HMO biosynthesis and degradation networks, respectively, are visualised using the Tulip software. Species possessing specific GH activities are represented as Krona diagrams.

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Human Milk: Oligo?Saccharides and More

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The first 1000 days, starting after conception, are a period of rapid growth and development with specific nutritional requirements. The nutritional influence begins parenterally in utero and is affected by the nutritional status and environment of the mother. After birth, human milk (HM) is the preferred nutrition. The composition of HM is uniquely tailored to infant's specific demands and is influenced by diet, genes, lifestyle and health of mother and child. WHO recommends exclusive breastfeeding (BF) for first six months, and continued BF with adequate complementary foods for up to two years and beyond. The development of the gastro-intestinal tract and its microbial ecosystem during early life is largely influenced by human milk (HM) tuning the infant's immune responsiveness and metabolism towards requirements. Human milk offers nutrients and other bioactive factors of which many are glycans and glycoconjugates (Newburg, 2013). More than thousand different HM proteins with hundreds of glycoproteins reflecting variable functional and nutritional needs (Zhu et al, 2020). The lipid fraction of HM is composed of variable (long-chain polyunsaturated) fatty acids as part of triglycerides, polar lipids (incl. glycolipids) within a complex milk fat architecture driving regulation of energy homeostasis and cognitive development and immunity. HM contains -as third largest fraction- human milk oligosaccharides (HMOS) with more than 200 identified complex molecular structures. Many of those glycans and glycoconjugates affect the microbiota and the developing immune system (Bode, 2015). Future studies on the causes and consequences of the variation in HM composition related to maternal nutrition, health and lifestyle will deepen our understanding of the nutritional needs of mothers and their young children during the first 1000 days. The structure-function-relation of free and bound glycans of human milk play a crucial role in our understanding of the optimal development of the infants during the first 1000 days (Georgi et al, 2013). State-of-the-art analytical methods are applied to different study cohorts to investigate the contribution of glycans and glycoconjugates to the observed clinical benefits (Dingess et al, 2021; Siziba et al, 2021; Eussen et al, 2021).

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Designing an In-Vitro Digestion Model By Using Novel Microbiome-Associated Enzymes to Study Glycan Function

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Glycans have long been known to have important metabolic, structural, and physical roles in biological systems. Humans consume dietary glycan sources from dozens of different plants and animals, many of which cannot be broken down by enzymes encoded in the human genome. Microorganisms utilize these indigestible glycans by microbial fermentation that act as nutrients. Most gut commensals participate in human digestion by functioning carbohydrate-active enzymes (CAZymes) such as glycosidases, which are utilized to cleave oligosaccharides to benefit both themselves and the host's health. Thus, glycans reach the colon, where they are metabolized by probiotics using their enzymes. In-vitro digestion models could be an excellent tool to investigate the interaction between microbial enzymes and glycans in laboratory conditions to better understand glycan digestion by microbial metabolism. However, due to the lack of human enzyme specificity, current in-vitro digestion models are not suitable for glycan studies. Accordingly, developing innovative models that include host and microbiome-associated enzymes is crucial for leading the way for glycan research.
In this study, unique glycosidases from several bacteria that predominate in the human digestive system were investigated. 32 distinct enzymes were recombinantly cloned and generated using a specific in-vivo Expresso Rhamnose Cloning and Expression System. The chosen enzymes were integrated into a standard in-vitro digestion model that solely included human-associated digestion enzymes. Finally, the new digestion model was tested on milk and plant-based glycoprotein sources and obtained *N*-glycans were analyzed by the MALDI-TOF-MS.
Numerous scientific studies can be performed with this novel model in terms of glycans and their utilization by microbial enzymes, which would be a critical step in the glycobiology field. This model also shed light on future studies in many other fields including the food industry, medicine, and pharmacy. Designing the first in-vitro digestive system by integrating microflora-derived enzymes will contribute to the glycobiology field in a simple, economical, and reproducible way to determine the bioavailability and bioaccessibility of bioactive glycans.

Acknowledgment

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CELL FACTORY ENGINEERING AND PROCESS DESIGN TO MAKE HUMAN MILK OLIGOSACCHARIDES (HMO) AVAILABLE TO THE WORLD.

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Key Words: Human Milk Oligosaccharides (HMO); E. coli K12;

HMOs are a unique class of compounds of human milk making up for approximately 10% of the total dry matter of human milk. Breast-feeding is always the ideal nutritional solution for infants, but not always possible. DSM/Glycom has worked hard on making HMOs available to close the gap between infant formula and breast milk and thereby creating a healthier life for formula-fed infants. There are many clinical proven benefits of adding HMOs to infant formula, including improved composition of the intestinal microbiota, strengthened immune function, lower infection frequency etc. These beneficial properties of HMOs make them ideal candidates for improving well-being and addressing health conditions beyond their known functions in infants.
Using an E. coli K12 manufacturing platform, metabolic engineering was the key to make HMOs commercially available in multi-ton scale. The strains were genetically engineered in numerous ways to secure a high-quality product. USP and DSP processes were developed and scaled-up to secure an economically viable manufacturing process. Using a holistic view of the manufacturing process from start to end, we have secured a steady supply of HMOs for the benefit of the world. DSM/Glycom has until now successfully scaled-up the production of 8 different HMOs to multi-ton scale production and are presently in the process of getting worldwide regulatory approval for all these great compounds for their use in infant formula.

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Mechanism and Engineering of *Bifidobacterium* Lacto-*N*-biosidase for Type I HMO synthesis

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Human milk oligosaccharides (HMOs) furnish breast-fed infants with a number of health benefits. Their unique composition, differing from other mammal's milk, drives active research to synthesize and produce the main HMO structures as supplements for infant formula milks. Various beneficial effects are critically important for early development and infant health, functioning as prebiotics and antimicrobial agents as well as exerting immunomodulation effects. Chemical synthesis, enzymatic synthesis (biocatalysis) and fermentation (cell factory) approaches are current focus of research (Faijes et al., 2019).

Lacto-*N*-biosidase from *Bifidobacterium bifidum* (LnbB) is a GH20 enzyme present in the gut microbiota of breast-fed infants that hydrolyzes lacto-*N*-tetraose (LNT), the core structure of the most abundant type I HMOs.

He we report on the structural and mechanistic features of *Bb*LnbB and its engineering into a transglycosylase for the enzymatic synthesis of LNT:

1.- Family GH20 glycoside hydrolases is mainly composed of N-acetylhexosaminidases that hydrolyse the non-reducing end GlcNAc or GalNAc residue from their glycan substrates, except for LnbB that releases the disaccharide LNB from the core LNT structure of type I HMOs. Structural analysis identifies different domain organizations within the GH20 family, with a particular multidomain organization for LnbB enzymes, where a lectin domain provides a remote element essential for activity (Val-Cid et al., 2015). QM/MM calculations of the retaining substrate-assisted mechanism of LnbB reveals a conformational itinerary $^{1,4}B/^{1}S_3 \longrightarrow [^{4}E]^{\ddagger} \longrightarrow {}^{4}C_1/{}^{4}H_5 \longrightarrow [^{4}E/{}^{4}H_5]^{\ddagger} \longrightarrow {}^{1,4}B$ featuring an oxazolinium ion intermediate, where a critical histidine (His263) switches between two orientations to modulate the pKa of the acid/base residue, facilitating catalysis (Cuxart et al., 2022).

2.- Engineering glycoside hydrolases into transglycosylases may provide biocatalytic routes to the synthesis of complex oligosaccharides. As compared with the wt LnbB enzyme with negligible transglycosylation activity, mutants at the donor subsites with residual hydrolase activity within 0.05% to 1.6% of the wild-type enzyme result in transglycosylating enzymes with LNT yields in the range of 10–30%. Mutations of Trp394, located in subsite -1 next to the catalytic residues, have a large impact on the transglycosylation/hydrolysis ratio, with W394F being the best mutant as a biocatalyst producing LNT with 32% yield (Castejón-Vilatersana et al., 2021).

A follow-up of this work will be the introduction of additional mutations in the acceptor subsites with the goal of adding beneficial binding interactions for the lactose acceptor that may result in even further enhanced TG efficiency.

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IMS-IMS coupled to cryogenic IR spectroscopy for the identification of human milk oligosaccharides

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While oligosaccharides, or glycans, play a fundamental role in many biological processes, their intrinsic isomeric heterogeneity presents a significant challenge to their analysis. This is further complicated by paucity of isomerically pure analytical standards. Cryogenic infrared (IR) fingerprinting is an emerging technique that promises unparalleled structural sensitivity (Warnke et al, 2021). It can unambiguously identify glycans for which reference fingerprints have previously been recorded to a database. In this work we demonstrate how the combination of ion mobility spectrometry (IMS) with mass spectrometry (MS) and IR fingerprinting can be used to identify isomers with a reduced need for isomerically pure standards. This is made possible through an IMS^2 technique, where fragments of mobility-separated oligosaccharides are identified using both IMS separation and IR fingerprints, allowing us to reconstruct the isomeric form of the parent molecule in a bottom-up approach (Bansal et al, 2022).

We begin with mobility separation of oligosaccharide isomers using structures for lossless ion manipulations (SLIM). After separation during one or more cycles along a 10 m serpentine path, we fragment the parent molecules by collision-induced dissociation (CID) directly on our SLIM device. We then send the fragments through a second stage of mobility separation before directing them to an ion trap, where we perform cryogenic IR spectroscopy to identify them. By identification of fragments diagnostic of the isomeric form of the parent molecule, we identify the latter using just a few standards to construct a fragment IR database. The IR spectrum of the parent is then added to our database for identification of fragments from still larger glycans.

We demonstrate our approach by the identification of isomeric human milk oligosaccharides (HMOs). Following the outlined strategy, we present proof-of-principle data to assign isomers of the hexasaccharide Lacto-N-difucohexaose (LNDFH), as well as of the heptasaccharide MFLNH, and the octasaccharide DFLNH. In each case, a small database of smaller standards and fragments thereof, is sufficient to identify the glycan backbone as well as the position of fucose residues. As we grow the database by adding isomer-selective IR fingerprints of the identified parent molecules, we can use it to identify isomers of still larger human milk oligosaccharides.

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Activated sugars for oligosaccharide synthesis

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Glycans in their unbound form and glycans linked to proteins, lipids and the cell surface have shown to exert a multitude of biological functions. Consequently, there is immense interest to synthesize and modify a wide range of these structures to investigate structure-specific functions, and to utilize them as nutraceuticals or to enhance the efficacy of biopharmaceuticals. Prominent examples for applications are the synthesis of human milk oligosaccharides that are added to infant food formulations or the *in-vitro* glycoengineering of *N*-glycans that are attached to therapeutic proteins like monoclonal antibodies. In comparison to the biotransformation of whole cells that only allows the synthesis of few glycans, *in-vitro* enzymatic synthesis using Leloir glycosyltransferases allows to synthesize a wide variety of structures [Rexer et al. 2021]. However, these syntheses rely on activated sugars as substrates. As of today, the price for activated sugars typically exceeds 1000 €/g and therefore prohibits any synthesis beyond milligram amounts.
Here, we report the development of cell-free enzyme cascades for the synthesis of the four major nucleotide sugars involved – UDP-galactose, GDP-fucose, CMP-Neu5Ac and UDP-GlcNAc – from inexpensive precursors. All cascades comprise a set of recombinant enzymes expressed in *E. coli*. To circumvent expensive enzyme purification, cell lysates containing the recombinant enzymes are used as the biocatalyst formulation. Moreover, to optimize biocatalyst loads and final product titers, Design of Experiments (DoE) approaches are used iteratively in combination with kinetic models. As examples, UDP-galactose and UDP-GlcNAc can now be produced with titers of 51 g/L and 61 g/L, respectively, in a batch time of 24 h while CMP-Neu5Ac can be produced with final titers of 57 g/L in 8 h [Mahour et al 2022]. In the future, the activated sugars and selected oligosaccharides will be made commercially available through our spin-off **eversyn®**.

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SESSION 4:
Glycoengineering of Biopharmaceuticals

Chair: Mike Butler

Keynote Lectures:

Glycosylation Challenges in Biopharmaceutical Manufacturing

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Antibody glycosylation and site-specific antibody-drug conjugates

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PK beyond FcRn: How glycans impact pharmacokinetics of therapeutic antibodies.

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Therapeutic antibodies (mAbs) have revolutionized the treatment of cancers and autoimmune diseases. One of their desirable properties is a long circulatory half-life. Interestigly, it has been shown that glycosylation can significantly impact pharmacokinetic (PK) properties of these immunoglobulin G1-based proteins. Especially, oligomannose and hybrid type glycans, whose occurrence is a typical difference between recombinant and natural antibodies, can notably increase mAb clearance (Falck et al 2020; Higel et al 2016). mAb glycosylation is routinely considered as a critical quality attribute with respect to its impact on effector functions, stability and immunogenicity. However, assessment of its criticality regarding PK behaviour is still hindered by limited knowledge.

In this talk, we give an overview of the knowledge collected by us and others over the past decade and highlight some recent discoveries. Glycoform-resolved PK analysis was performed in various animal models as well as in humans. In a recent porcine study, we discovered differences between intravenous and subcutaneous injections with regard to glycoform-driven effects. Even more than for classical mAbs with only Fc glycosylation, the differences between glycoforms are divers and pronounced in Fab-glycosylated antibodies and Fc-fusion proteins (Higel et al 2016).

An open question remains which mechanisms drive the glycoform preference in the clearance of mAbs. Glycoform preferences in FcRn binding have been reported which, however, are so small compared to oxidation effects that a significant PK impact is hard to imagine (Wada et al. 2019). Mannose receptor and Asialoglycoprotein receptor, two C-type lectins with far more pronounced glycan binding preferences, have been suggested as mediators, as they are involved in clearance of glycoproteins in the liver. However, evidence is sparse and often indirect. We are currently exploring these pathways with receptor-deficient animal models in natural and therapeutic settings. Interestingly, we have various indications that the role of the Asialoglycoprotein receptor is much more limited than is widely assumed.

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Sculpting therapeutic monoclonal antibody *N*-glycans using endoglycosidases

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Immunoglobulin G (IgG) monoclonal antibodies are a prominent and expanding class of therapeutics used for the treatment of diverse human disorders. The chemical composition of the *N*-glycan on the fragment crystallizable (Fc) region determines the effector functions through interaction with the Fc gamma receptors and complement proteins. The chemoenzymatic synthesis using endo-beta-*N*-acetylglucosaminidases (ENGases) emerged as a strategy to obtain antibodies with customized glycoforms that modulate their therapeutic activity. We discuss the molecular mechanisms by which ENGases recognize different *N*-glycans and protein substrates, especially those that are specific for IgG antibodies, in order to rationalize the glycoengineering of immunotherapeutic antibodies, which increase the impact on the treatment of myriad diseases.

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Implementation of in vitro glycoengineering of monoclonal antibodies into downstream processing

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In vitro glycoengineering using exoenzymes for specific modification is recognized as appropriate method to tailor sugar moieties of glycan structures during the recombinant production of monoclonal antibodies (mAbs). Enhanced in vitro glycoengineering approaches using β1,4-galactosyltransferase and α2,6-sialyltransferase are described to improve the efficiency of galactosylation and sialylation with the aim to implement in vitro glycoengineering into common mAb purification processes.

Specifically, the reactions of β1,4- galactosyltransferase and α2,6-sialyltransferase were shown to work on column during affinity chromatography using Protein A or KappaSelect, the latter providing more efficient galactosylation and sialylation. Performing in vitro glycoengineering on column enabled the use of cell culture harvest that yielded results comparable to those of purified bulk. Based thereon, an optimized two-step mixed mode approach was found most appropriate to integrate in vitro glycoengineering into the overall manufacturing process. Using fermentation harvest for on-column reaction of β1,4-galactosyltransferase combined with in-solution reaction of α2,6- sialyltransferase, this approach proved to be highly efficient with regard to galactosylation and sialylation reaction. Moreover, the enzymes applied in in vitro glycoengineering could be separated, recycled and reused in further reactions to improve economic efficiency. Overall, the study provides a toolbox for in vitro glycoengineering and presents an optimized easy-to-handle workflow to implement this method into the downstream processing of industrial mAb production.

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Multiplexed effector function assessment of antibody glycoforms using ACE-MS

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Monoclonal antibodies (mAbs) consist of variable domain, interacting with the antigen, and a constant domain, necessary for an immune response via Fcγ receptors, as well as for mAb half-life by interaction with the neonatal Fc receptor (FcRn). Therefore, it is important to study the affinity of mAbs and their proteoforms with these receptors. Common approaches, such as SPR, provide an overall affinity response for all different mAb proteoforms rather than in a proteoform specific manner.
In this presentation, we want to show an innovative approach based on sheathless CE-MS to study relative affinities of different mAb proteoforms with the FcRn and FcyRIIa receptor. To determine the affinities, first the CE capillary was filled with the FcR followed by the injection of a mAb samples. The separation was performed using ammonium acetate pH 6 or 6.8 for FcRn or FcyRIIa, respectively. We will show that we are able to determine the affinity of mAb proteoforms as a consequence of their different mobility shifts, using different amounts of FcR in the background electrolyte. Hyphenation to MS allowed us to detect the mAb alone as well as in complex with one or two FcRs.
FcRs form commonly higher order complexes with mAbs at cell surfaces in vivo. Our liquid phase approach allows to reflect this in vivo situation better compared to techniques like affinity LC or SPR, which use immobilized receptors. Overall we think that our approach will tremendously boost the study of interactions between mAb proteoforms and FcRs. This is very important for the development of new drugs able to activate only a certain immune response in our body.

GalMAX: Model-inspired glycoengineering for biopharmaceutical quality assurance

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β4-galactosylation is a critical quality attribute (CQA) of monoclonal antibodies (mAbs) because of its inherent heterogeneity and influence on the oncolytic activity of these biopharmaceuticals (Planinc et al, 2017; Liu, 2017). mAb β4-galactosylation variability arises from both metabolic and cellular machinery bottlenecks: (i) limited availability of uridine diphosphate galactose (UDP-Gal) and (ii) reduced availability/activity of β-1,4 galactosyltransferase (β4GalT), the enzyme which catalyses β4-galactosylation. Here we present GalMAX, a simple and robust cell engineering strategy that simultaneously eliminates the above bottlenecks to maximise mAb β4-galactosylation and address the quality assurance challenges posed by this CQA.

A compact metabolic model for CHO cells, which accounts for nucleotide sugar demand towards cellular and product glycosylation, was used to identify serine (O)-linked cellular glycosylation as a major sink of UDP-Gal (de Val et al, 2021). CRISPR-Cas9 was used to abrogate cellular O-galactosylation by knocking out the core 1 β3-Gal-T-specific molecular chaperone (COSMC) from two mAb-producing CHO cells, CHO-DP12 and CHO-VRC01. COSMC knockout cells were enriched using lectin-aided fluorescence cell sorting and transfected with a plasmid containing the human β4GalT1 gene to produce DP12 and VRC01 GalMAX cells. COSMC-KO, β4GalT1+, and GalMAX variants of DP12 and VRC01 were cultured in batch and fed-batch mode; mAb glycoprofiling was performed with LC-MS (Carillo et al, 2020).

Under batch cultivation, the DP12-GalMAX cells produced 96% galactosylated mAb glycoforms (40% above parental cells), and the VRC01-GalMAX cells yielded 98% galactosylated species (2.2-fold increase over parental cells). Under fed-batch culture, DP12-GalMAX achieved 92% galactosylated glycoforms (14.7% higher than DP12-GalT+ cells), and VRC01-GalMAX produced 95% galactosylated glycoforms (11% higher than VRC01-GalT+ cells). The differences observed when comparing GalMAX cells with the GalT+ variants demonstrate how the COSMC knockout channels UDP-Gal consumption towards mAb β4-galactosylation. The cell engineering events had no negative impact on cell growth, metabolism, or mAb titre. By yielding high levels of β4-galactosylation, our model-inspired glycoengineering strategy reduces product variability and has the potential of enhancing mAb oncolytic activity. Our GalMAX platform has also been used to develop strategies for real-time control of mAb glycosylation and for facile and robust mAb glycoprofiling based on lectin-aided flow cytometry measurements. GalMAX has great potential in contributing to biopharmaceutical quality assurance.

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Modelling the processing of glycans on secreted glycoproteins

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N-glycosylation is known to be important for the pharmacological properties and efficacy of many therapeutic proteins (also known as biologics). However, glycosylation is a complex and non-templated process which often results in a highly heterogeneous population of glycan structures, preventing the precise control over the resulting glycan distribution. Therefore, a method to rationally engineer biologics with increased homogeneity and advantageous glycan structures would be highly beneficial to the pharmaceutical industry.

A computational model of glycan biosynthesis, which combines stochastic simulation with Bayesian fitting, has been experimentally validated to provide meaningful insights into the organisation of the glycosylation machinery when simulating the biosynthesis of the total N-glycan repertoire of the cell[1]. The project detailed here aims to use this computational modelling tool to investigate the relationship between organisation of the glycosylation machinery in the Golgi and the glycoform distribution of a model biologic.

We use the modelling to investigate the relationship between whole cell (WC) glycosylation and the glycosylation of the model biologic Herceptin. Glycan profiles from both WC and Herceptin have been acquired in two CHO cell lines: WT and a mutant with altered glycosylation. The computational model predicts how the glycosylation machinery changes between WT and mutant to generate the mutant's altered glycan profile. We will model altered WC glycosylation of the mutant relative to WT, then apply the predicted changes of the glycosylation machinery to the WT Herceptin glycan profile. By assessing how well the Herceptin glycan profile predicted this way matches with experimentally determined one, will allow us to conclude contributions to the glycosylation of a monoclonal antibody therapeutic by both the glycosylation machinery and the protein's structure itself. This information could be used to establish methods to refine computational predictions for controlling biologic glycosylation.

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Effector function evaluation of therapeutic antibodies with defined and homogeneous Fc N-glycans

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N-glycosylation at the fragment crystallizable (Fc) region of immunoglobulin G (IgG) antibodies is a conserved modification of structural and functional importance. Since it impacts antibody-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP), the N-glycan structure is important to take into consideration when developing therapeutic antibodies. However, the structural heterogeneity of the Fc N-glycans complicates analysis and regulation. By combining a chemoenzymatic engineering technology for Fc-specific N-glycan remodeling (Huang *et al*. 2012) and cell-based Fc effector activity bioassays for functional evaluation (Lallemand *et al*. 2017), we here demonstrate fast, specific, and accurate testing.

The technology for generation of homogeneous N-glycans comprises two enzymatic steps starting with Fc-specific deglycosylation to the core GlcNAc, with or without fucose, followed by transglycosylation using oxazoline-activated N-glycans. For the generation of core-afucosylated structures, an exo-α1,6-fucosidase was included during the deglycosylation step. This technology is considerably faster and more efficient than, for example, cellular engineering or cell-free engineering using hydrolases, transferases, and nucleotide sugars.

For effector function evaluation, cell-based reporter gene bioassays utilizing engineered target and effector cells were used. Upon antibody binding to the target cell antigen and effector cell FcγR receptor, a cascade reaction in the effector cell is initialized resulting in luminescence proportional to the functional activity of the antibody. The assays resemble the natural conditions to a greater extent than ligand-based assays, offer less variability than other cell-based assays, and are at the same time fast and accurate, providing easily interpreted results. Here, we generated twelve variants of a therapeutic antibody, each variant homogeneously remodeled with one of the common complex biantennary N-glycan structures with or without core fucose, and evaluated their effector functions. Our data displayed distinct trends regarding core-fucosylation, sialylation, and galactosylation, including differences between the monogalactosylated isomers. Taken together, the use of high-quality systems for antibody N-glycan remodeling and effector function evaluation will gain valuable knowledge about the relationships between Fc N-glycosylation and effector functions, and facilitate such analyses during monoclonal antibody development.

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Tuning the quality of a biopharma product by choice of cell line up to media optimization

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Product titer and quality criteria are the main aspects to be considered in the design of biopharmaceutical protein production processes as each biopharmaceutical has individual properties and requirements. The product quality and thus the potency of a protein may vary greatly from one used cell production system to another. In particular post-translational modifications like glycosylation of biopharmaceuticals can be of crucial importance for bioactivity, efficacy and half-life time. Therefore, the selection of the most suitable expression host is crucial to achieve the desired product characteristics. Here we present the advantage of using different expression platforms, using FyoniBio's mammalian host cell systems CHOnamite® and the human GEX® platform as examples, for the development and production of a certain biopharmaceutical and biosimilars at the desired quality. Two case studies demonstrate the versatility of both expression hosts in product glycosylation and opportunities for bioprocess optimization.

Influence of peptones on CHO glycosylation profiles

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Efforts in upstream process improvement primarily focus on optimizing CHO cell growth and protein production. However, appropriate glycosylation of monoclonal antibodies (mAb) is critical for their safety and efficacy. Modification of glycosylation profiles can affect serum half-life, recruitment of immune effector cells, stability, cytotoxicity, anti-inflammatory properties, and antigen affinity. Glycosylation patterns vary amongst different host cell lines and can further be influenced by upstream process parameters, including dissolved oxygen levels, culture temperature, pH, and nutrient availability. For instance, a limitation of glutamine or glucose can lead to changes in sialylation, as well as hybrid and high-mannose glycans. Here, we investigated how nutrient availability and complexity affect glycosylation profiles of mAb produced with CHO-K1, CHO-K1 GS, and DG44 cell lines. Evaluation of different chemically defined cell culture media and feeds led to minimal changes. Addition of plant-derived peptones led to pronounced alterations in mAb glycosylation, enabling modulation of glycan structures to achieve a desired biosimilar profile. While supplementation with soy peptones led to an increase in more differentiated glycans and decrease in acidic charge variants, addition of cotton and wheat peptones increased less-differentiated glycans and did not alter the charge variant profile. Thus, targeted supplementation of CHO cell cultures with peptones can improve productivity and glycosylation profiles.

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SESSION 5:
Tools & Technologies for Glycoanalytics and Glycobioinformatics

Chair: Falk Büttner

Keynote Lecture:

Glycans at the frontiers of chronic inflammation, autoimmunity and cancer: mechanisms and clinical implications

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High-throughput glycosylation profiling applied to the analysis of Covid-19 virus evolution and changes in human serum following infection

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National Institute for Bioprocessing Research and Training, Dublin, Ireland

A method for quantitative and rapid analysis of glycan profiles of glycoproteins or biotherapeutics is required for the determination of changing patterns of heterogeneous glycosylation. Such changes can be monitored through isolation and labelling of glycans derived from multiple biological samples. Hydrophilic interaction liquid chromatography (HILIC-HPLC or UPLC) has become a standard method of separation and analysis but often the workflow for glycan derivatisation is time-consuming. We present an innovative streamlined 96-well-plate-based platform utilizing InstantPC for glycan derivatization that is rapid and sensitive. Assignment of glycan structures is based on GU values that we have incorporated into a database from known standard structures.

We have applied this analytical protocol to N-glycan profiling of serum from a cohort of Covid-infected patients and to monitor the evolutionary changes that occurred to SARS-CoV-2 viral spike protein variants. Use of this platform revealed both similarities and differences between the serum N-glycomic fingerprints of COVID-19 positive and control patient cohorts. Such serum N-glycomic changes may indicate or correlate to the changes in serum glycoproteins upon Covid infection and could have potential use in clinical surveillance. We also investigated quantitatively the N-glycosylation profiles of seven major emerging spike variants of the Covid virus. The aim was to understand the changing pattern of N-glycan profiles in SARS-CoV-2 evolution in addition to the widely studied amino acid mutations. Different spike variants exhibit substantial variations in the relative abundance of different glycan peaks and subclasses. This provides a valuable framework for quantitative N-glycosylation profiling of new emerging viral variants and give us a more comprehensive picture of COVID-19 evolution.

Detection and quantification of α-Gal epitopes in intact monoclonal antibodies by NMR spectroscopy

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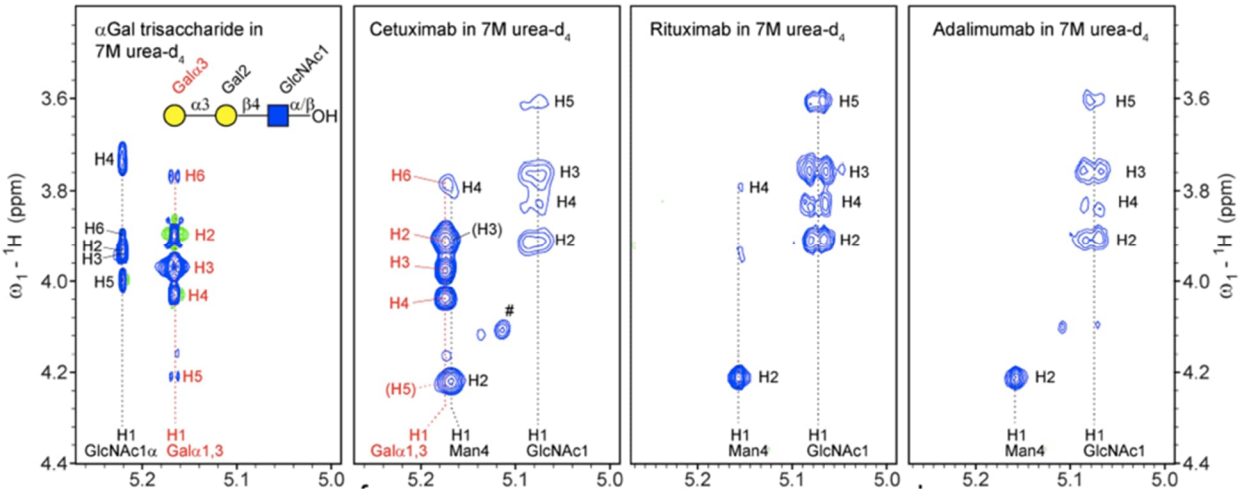
The terminal trisaccharide Galα1,3Galβ1,4GlcNAc, also called α-Gal or xenotransplantation epitope, exposed on cell or protein surfaces can cause severe immune reactions in humans, such as hypersensitivity reactions or even anaphylactic shock. Therapeutic proteins expressed in murine cell lines may contain α-Gal epitopes, and therefore their absence or presence needs to be tightly monitored to minimize any undesired adverse effects. The analytical identification of α-Gal epitopes in glycoproteins using the common standard techniques based on HPLC coupled with MS is challenging, mainly due to the isobaricity of hexose stereoisomers. Here, we present a straightforward NMR approach to detect the presence of α-Gal in biotherapeutics based on a quick screen with sensitive ^{1}H-^{1}H TOCSY spectra followed by a confirmation using ^{1}H-^{13}C HSQC spectra (Hinterholzer et al. 2022).



The spectra are measured in 7 M urea, which overcomes the size limitation of NMR spectroscopy leading to sharp lines and simplified spectra. The sensitive 2D ^{1}H-^{1}H TOCSY spectrum, which can be measured in few hours, seems to be quite competitive in detecting the immunogenic glycoepitope α-Gal (see Figure) and can even detect low amounts like in the Fc-fusion protein abatacept, which is produced in CHO cells.

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Correcting for sparsity and interdependence in glycomics to enhance discovery, diagnosis, and drug development

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Glycans are fundamental cellular building blocks, involved in many organismal functions. Advances in glycomics are elucidating the essential roles of glycans. Still, it remains challenging to properly analyze large glycomics datasets, since the abundance of each glycan depends on many other glycans that share many intermediate biosynthetic steps. Furthermore, the overlap of measured glycans can be low across samples. We address these challenges with GlyCompare, a glycomic data analysis approach that accounts for shared biosynthetic steps for all measured glycans to correct for sparsity and non-independence in glycomics, which enables direct comparison of different glycoprofiles and increases statistical power. We demonstrate the power of decomposing glycoprofiles to their substructures using GlyCompare in a few case studies where we analyze diverse N-glycan profiles from glycoengineered erythropoietin and identify biologically meaningful groups of mutant cell lines through clustering of glycoprofiles, and boost statistical power to classify normal and cancer samples based on their N-linked and O-linked glycomes. We have further deployed this approach to aid in structural annotation of glycoproteomic data, to identify interactions between glycan structures and their underlying protein structures, and to aid in glycoengineering of biologics. To facilitate the use of this method for glycomics data analysis, we further introduce GlyCompareCT, a portable command-line tool that allows anyone to deploy these advanced. Thus, this new paradigm of a substructure-oriented approach, along with easy-to-use software, will enable researchers to take full advantage of the growing power and size of glycomics data.

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New avenues for biomarker discovery in human blood plasma via an improved in-depth analysis of the low-abundant *N*-glycoproteome

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To understand the molecular implications of protein glycosylation and to leverage those insights for clinical diagnostic and biopharmaceutical production innovative glycoproteomic technologies are required (Kirwan et al 2015; Zhou et al 2019). Essential elements in this regard are the site-specific identification, structural characterization, and quantification of the protein glycosylation. Recently significant advances were made particularly towards structure-focused *N*-glycoproteomic analyses. The mass spectrometric analysis of intact *N*-glycopeptides using stepped collision fragmentation along with glycan oxonium ion profiling, for instance, now enables to reliably discriminate between different *N*-glycan types and to discern isobaric structural features such as antenna- and core-fucosylation (Hoffmann et al 2018).
Still, there are some weak points current *N*-glycoproteomic approaches are affected by. Among the most significant are: 1) handling of incorrect identifications 2) identification of rare and modified *N*-glycans such as sulfated *N*-glycans 3) insufficient glycoproteomic coverage particularly in complex samples such as human blood plasma.
To address these shortcomings, we have developed an innovative *N*-glycoproteomic workflow that aims at providing comprehensive site-specific and structural *N*-glycoproteomic data on human blood plasma glycoproteins – primarily derived from the middle and lower concentration range. To achieve this, the workflow features protein enrichment and fractionation strategies, the use of high-resolution mass spectrometry with stepped collisional energy fragmentation, and an extended set of marker ions that were included into the glycan oxonium ion profiling. In addition, the workflow also covers a new data validation strategy by including a semi-automated decision tree procedure.
With this workflow we were able to advance in the analysis of the human blood plasma *N*-glyco-proteome by being able to analyze human blood plasma glycoproteins that have concentrations as low as 101 pg/mL (Nanjappa et al 2014). Furthermore, we could significantly improve the description of the *N*-glycan micro-heterogeneity by including rare *N*-glycans such as sulfated and glucuronidated ones. Our analysis also includes the confident differentiation of ambiguous *N*-glycan structures like bisecting GlcNAc or antenna- vs core-fucosylation. In total 1929 *N*-glycopeptides and 942 *N*-glycosites derived from 805 human middle to low abundant glycoproteins were identified.
Overall, the presented workflow holds great potential to increase our understanding of protein glycosylation and to foster the discovery of blood plasma biomarker.

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Entering the N-glycan structure jungle from two sides

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The enormous structural diversity accomplished by just a few building blocks and enzymes poses a huge analytical challenge. Simple systems such as IgG can be tackled with basically "one-dimensional" methods such as HILIC-HPLC with fluorescence or mass spectrometric (MS) detection. Choice of a shape-selective stationary phase, *i.e.* porous graphitic carbon (PGC), coupled to MS provides superior isomer separation. Individual peaks are characterized by their (negative mode) fragment spectrum and their retention time – the latter, however, is prone to some fluctuation. We propose normalizing retention times with a tightly spaced Time Grid (glyco-TiGr) of isotope-labeled internal standards. Using recombinantly expressed glycosyltransferases, a library of normalized retention times covering around 140 structures has so far been built. Of that, not less than 40 isomeric structures consisting of five hexoses, four *N*-acetylhexosamines and one fucose residue were generated. Notably, this endeavour resulted in the detection of novel bisected N-glycans in brain (Helm et al, 2022; Helm et al, 2021).
Predictably, even the most skillful application of PGC-LC-MS will become entangled in the impenetrable "jungle" of fucosylated and sialylated tri- and tetraantennary structures. Irrespective of advances in separation science, isomeric large glycans will not be sufficiently separable to allow for unambiguous identification by retention time or/and negative mode MS/MS. Even worse, the results of glycomic studies usually take the shape of exceedingly long lists that are almost incomprehensible to the human mind. A remedy to this problem could be found in the comprehensive characterization of critical glycan epitopes by breaking down large glycans into smaller elements of limited diversity. Chemical degradation of a sample „distills" the almost infinite number of distinct glycan structures of a given N-glycome to a tractable number of smaller oligosaccharide fragments, which are unambiguously identified by their masses and specific retention times on PGC. Thus, even highly complex glycan mixtures can be characterized by a comprehensible glycan-feature foot print.

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New approach using Online SPE purification and procainamide for HILIC-FLR-MS N-glycan analysis

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Glycosylation is one of the most prominent post-translational modification of proteins that plays a crucial role in maintenance of the structure and protein activity. In the last decades, monoclonal antibodies (mAbs) continue to reign supreme the biopharmaceutical approvals due to their successful treatment of a vast array of serious diseases, such as cancers, immune disorders, and infections. mAbs are glycoproteins and the modifications in the monosaccharide residues may alter their functioning such as their half-life, immunogenicity, toxicity, stability, and solubility. Therefore, special attention should be paid to the detection of changes in the glycosylation patterns. The main goal of our study was to develop and validate a rapid sample preparation using procainamide labelling and online SPE purification to improve monitoring of N-glycans and compare the procedure with other commercial kits. This analytical approach will be used to monitor batch to batch sample of glycosylated biotherapeutics but also to investigate and to understand the impact of N-glycosylation alteration at several diseases like endometrioses and human immunodeficiency virus (HIV).
Briefly, N-glycans were (i) enzymatically released using PNGase F, (ii) labelled with procainamide or commercial kits: GlycoWorks RapiFluor-MS N-Glycan Kit[1] (RFMS kit) or Instant PC Kit, (iii) cleaned with µHILIC SPE plate or online HILIC SPE purification and (iiii) analyzed by HILIC coupled to a high-resolution mass spectrometer detector and a fluorescence detector.
Comparing the two approaches (online HILIC SPE purification and on the bench, purification using the µHILIC SPE plate) we proved that the online purification offers a higher sensitivity and abundance for the released N-glycans beside the better repeatability and the time saving. All these advantages came with accuracy and cost-effective.. Our protocol showed a higher sensitivity for the MS signals with a slight lower FLD signals comparing to the RFMS and the instant PC. We also tested the NIST mAb standard to compare our method to published results and we obtained the same observed ratio of galactosylation, sialylation, and fucosylation than described in literature. This protocol was actually used to characterize batch to batch variation of biopharmaceutical (Trixuma) and also applied to investigate N-glycans modification in endometrioses.
The described approach offers a reliable and repeatable method for released N-glycans analysis with a high sensitivity and a cost-effective manner. It showed its efficiency in analysis of biosimilars and antibodies from clinical samples

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Overcoming Unspecific Adsorption: LC-Analysis of Sialylated and Phosphorylated N-Glycans, Glycopeptides or Glycoproteins

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When separating sialylated and phosphorylated glycans, glycopeptides or glycoproteins with high performance liquid chromatography (HPLC), stainless steel may cause deleterious effects: Metallic surfaces in capillaries, column frits and bodies may lead to peak distortion, reduced recovery or even complete loss of the analytes. A novel covalent surface modification mitigates these unwanted interactions by a hybrid organic-inorganic surface. We demonstrate improved performance in HILIC, ion-pair reversed phase mode or mixed mode (anion exchange + reversed phase).
Equally, sample vial surfaces can vary in composition and purity of material which can impact adsorption as well as stability. We demonstrate how sample vials with a modified polypropylene surface increase consistency and recovery of released glycans and phosphoglycans.

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Methodical approach to design and optimize microarrays for efficient and high-throughput glycoprofiling and application in cancer sera glycoprofiling

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Glycosylation being a canonical posttranslational modification in eukaryotes is a key cellular mechanism regulating several physiological and pathological functions. Microarray has been the sole analytical platform, since their development in the past two decades, for analyzing carbohydrate-mediated recognition events in a high-throughput manner. Depending on the configuration i.e., lectin or glycan/glycoptotein which is immobilized on the slide surface, microarrays for glycomics studies can be broadly categorized as lectin and glycan/glycoprotein arrays respectively. Glycan/glycoprotein arrays have been vastly adopted to determine glycans specificities of glycan-binding proteins (GBPs) or glycoprofile samples in a high-throughput manner principally using GBPs. Each microarray consists of a large number of distinct glycans or glycoprotein samples immobilized on a solid substrate in well-localized discrete spots. Identification of presented glycan structures is performed by incubating with probes, mostly lectins, able to identify specific glycan moieties. However, several factors influence the final readout of these binding events ranging from concentration of the samples, spotting parameters, incubation parameters as well as the scanner parameters like pixel size, scan speed and most importantly the voltage applied to the photomultiplier tubes (PMT). Here we demonstrate the influence of some of these parameters on the results of the binding events with a goal to find appropriate parameters for performing reliable glycan/glycoprotein microarray analysis. The optimized microarray procedures were then applied in glycoprofiling of serum and depleted serum samples of more than 200 patients diagnosed with different types of cancer as well as for glycoprofiling of patients' serum samples diagnosed certain types of cancer before and after cancer treatment.

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Imaging Glycosylated Molecules One-At-A-Time

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Glycans (a.k.a carbohydates) are the most abundant biomolecules on Earth – playing key roles in intercellular signaling and energy storage. Among all classes of biomolecules, structural elucidation of glycans remains a difficult problem that hinders efforts to understand glycan properties. Ensemble-averaged methods to elucidate glycan structures are met with difficulties due to the high flexibility and complexity of glycans.

Here we bypass these limitations by using scanning tunneling microscopy (STM) to image single glycan molecules on surface, landed using electrospray ion-beam deposition (ESIBD) technique. Imaging a single glycan molecule directly reveals how its constituent monosaccharide subunits connect to one another i.e. its primary structure (Wu et al, 2020). Imaging multiple structures of a glycan reveals its shapes i.e. its secondary structure and its flexibility at the single linkage level (Anggara et al, 2020; Anggara et al, 2021).

We have recently extended our methods to reveal the structures of glycoconjugates (i.e. glycans covalently attached to peptides, lipids, or proteins) one-molecule-at-a-time, starting from simple glycopeptides and glycolipids to complex glycosaminoglycans and glycoproteins. Imaging glycoconjugates one-at-a-time visually determines all glycoforms (i.e. variants of biomolecules glycosylated with different glycan structures) present in a glycoconjugate sample.

Our approach permits the structures and mechanical properties of glycans to be determined for any molecule that can be electrosprayed.

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Biophysics, super-resolution microscopy, and bioengineering reveal structure-function relationships in glycocalyx biology

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Glycans are one of the four fundamental biopolymers that make up all cellular life. In humans, glycans are especially prominent on the extracellular side of the cell membrane. Estimates hold that virtually all secreted and cell-surface proteins and about half of cell-surface lipids are modified with glycans (Chandler & Costello, 2016; Steentoft et al, 2013). The collection of such extracellular glycoconjugates is termed the “glycocalyx” or literally translated “sweet cloak”. Thus, the glycocalyx is the first component of any cell to interact with the environment, be it other cells, proteins, nutrients, or pathogens (Möckl, 2020).

Despite this prominent location, the relevance of the glycocalyx had been overlooked for many decades. Fortunately, recent findings have brought glycans into the limelight. We now know that extracellular glycans are functionally involved in key cellular processes with substantial clinical impact, e.g. immune system regulation or cancer progression (Möckl et al, 2019; Wisnovsky et al, 2021; Pinho & Reis, 2015; Manni & Läubli, 2021).

Nevertheless, compared to e.g. protein or chromatin biology, our understanding of the functional role of extracellular glycosylation is still very limited. In order to answer the key question “How does the glycocalyx regulate, and how is it regulated by, cellular state?”, we require innovative approaches to the problem.

In my talk, I will summarize several topical key discoveries that underscore the relevance of the glycocalyx in cell biology. Then, I will give an overview of our recent research efforts in this area. In our studies, we combine state-of-the-art methods ranging from biophysics and biochemistry to optics and bioengineering. We leverage multiple orthogonal techniques such as super-resolution microscopy, iSCAT-based single-particle tracking, and real-time deformability cytometry in order to unravel the functional role of the glycocalyx in health and disease, which can be expected to unlock novel approaches for therapeutic intervention.

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Clinical glycomics for the diagnosis of congenital disorders of glycosylation

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Congenital disorders of glycosylation (CDG) refer to a heterogeneous group of diseases caused by inborn defects in various steps along *N*- and *O*-glycosylation pathways. Because the symptoms caused by CDGs are rather unspecific, clinical diagnosis of this disease group is difficult. Often, genome sequencing by next-generation sequencing technologies is required to detect genetic defects of glycosylation-linked enzymes for diagnosis.
To overcome these issues, we present ways to fast and reliably detect CDGs by multiplexed capillary gelelectrophoresis with laser-induced fluorescence detection (xCGE-LIF) with only one droplet of blood. In particular, we demonstrate the potential of the method on a female patient bearing a CDG manifested by mutations of the mannosyl-oligosaccharide glucosidase (MOGS) gene. This defect results in a decreased activity of the enzyme glucosidase I, causing a systemic change in *N*-glycosylation. The straightforward xCGE-LIF-based glycoprofiling analysis revealed an aberrant *N*-glycosylation, which is exclusively present in the patient, but not in healthy relatives. The aberrant *N*-glycan precursors $Glc_3Man_{7\text{-}9}GlcNAc_2$ could be found inside the patient's blood serum and on serum derived proteins like IgG. Furthermore, the accumulation of the free tetrasaccharide *Glc(α1-2)Glc(α1-3)Glc(α1-3)Man* could be observed in the patient´s blood. This tetrasaccharide is the product of an alternative bypass of the glucosidase I dependent *N*-glycan processing via endo-α-1,2-mannosidase. Detection of this tetrasaccharide using xCGE-LIF might in the future serve as a biomarker as part of patient screening during diagnostic work-up.

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GLYFINDER AND GLYCOPROTEIN BUILDER: ONLINE TOOLS FOR FINDING AND MODELING GLYCOPROTEINS IN THE PDB

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Tens of thousands of 3D structures of oligosaccharides have been deposited into the Protein Databank (PDB), representing hundreds of thousands of hours of effort by crystallographers. Yet, despite the critical importance of these structures in furthering the development of glycomimetic drugs, in explaining the activity of glycan-processing enzymes, and in providing a deeper understanding of the properties of glycoproteins and vaccines, they remain unnecessarily difficult to locate within the PDB. Part of this is due to limitations in searching for oligosaccharides on the PDB website, even after a recent carbohydrate remediation project completed by the PDB. While several databases have been reported that contain carbohydrate structural information extracted from the PDB, few offer flexible search capabilities and even fewer provide independent assessment of data quality.

Here we present the GlyFinder and GlyProbity webtools (glycam.org/gf) and illustrate their application to locating oligosaccharides, carbohydrate derivatives, and glycoproteins stored in the PDB. We highlight the utility of curating the data on the basis of the theoretical conformational (CHI) energies of the glycosidic linkages and illustrate how the deposited data can be employed to generate 3D models of glycoproteins, including the SARS-CoV-2 Spike protein (Nivedha et al, 2013; Grant et al, 2020).

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ABSTRACTS: POSTERS

Glycosciences – Fundamentals and Advances in Clinical and Biopharmaceutical Research
(A1 – A23)

Novel Emerging & Enabling Systems in Glycobiotechnology
(B1 – B19)

Synthesis and Function of Human Milk Oligosaccharides
(C1 – C4)

Glycoengineering of Biopharmaceuticals
(D1 – D12)

Tools & Technologies for Glycoanalytics and Glycobioinformatics
(E1 – E14)

Glycosciences – Fundamentals and Advances in Clinical and Biopharmaceutical Research

A1) Structural and functional analysis of ALG9: an endoplasmic reticulum α1,2-mannosyltransferase

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N-linked glycosylation is essential in eukaryotic organisms where the resulting glycoproteins are integral to myriads of specialized biological processes (Varki, 2017). Assembly of the *N*-linked glycan is catalyzed by the Asparagine-Linked Glycosylation (ALG) enzymes with monosaccharide transfer to the growing lipid-linked oligosaccharide (LLO) occurring on both sides of the endoplasmic reticulum membrane (Breitling & Aebi, 2013). Despite the ALG proteins being crucial to *N*-linked glycosylation, their mechanisms of substrate specificity, including glucose versus mannose donor substrate selection as well as the prevention of futile donor substrate hydrolysis, remain elusive. Particularly, among the ALG proteins, ALG9 stands out for its ability to catalyze mannose transfer to two branches of the maturing *N*-linked glycan and for its role in several cases of congenital disorders of glycosylation (CDG) in humans (Frank & Aebi, 2005; Haeuptle & Hennet, 2009). Using cryo-EM we solved the first structures of ALG9, an α1,2-mannosyltransferase and were able to obtain maps with ALG9 in complex with chemoenzymatically synthesized acceptor and donor substrates at 3.0 to 3.6 Å resolution. By visualizing the ALG9 ternary complex directly prior to mannose transfer, we identify the substrate binding sites while also inferring the mechanism of mannose transfer. Additionally, comparison of the ternary structure and donor substrate bound binary structure suggests how futile donor substrate hydrolysis is avoided. Combined with functional assays this structural information gives insight into how ALG9 has the required substrate specificity to synthesize the correct LLO needed for *N*-linked glycosylation as well as furthering a molecular understanding of how ALG9 mutations cause CDG in humans.

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A2) *In Situ* N-Glycosylation Signatures of Epithelial Ovarian Cancer Tissue as Defined by MALDI Mass Spectrometry Imaging

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The particularly high mortality of epithelial ovarian cancer (EOC) is in part linked to limited understanding of its molecular signatures. We implemented MALDI mass spectrometry imaging (MALDI-MSI) in combination with sialic acid derivatization in formalin-fixed paraffin-embedded tissue microarray specimens of less common EOC histotypes, namely low-grade serous, clear cell (CCC), endometrioid, mucinous histotypes as well as non-malignant borderline ovarian tumor (Grzeski et al, 2022). α2,6- and α2,3-sialylated N-glycans were enriched in tissue regions corresponding to tumor and adjacent tumor-stroma, respectively.

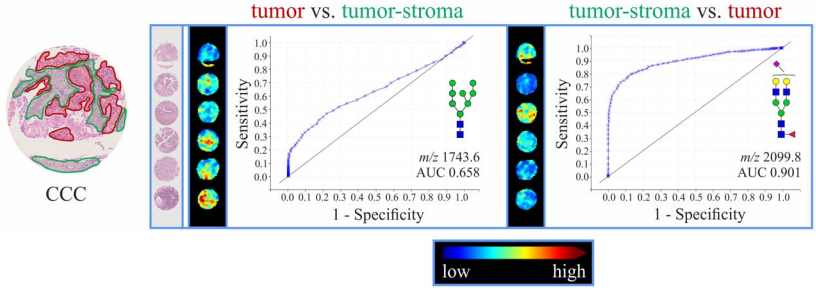

Figure 1. ROC curves and MALDI-MSI pictures of the most discriminatory N-glycan structures as determined for tumor and tumor-stroma in CCC TMA specimens.

Interestingly, analogous N-glycosylation patterns were observed in tissue cores of BOT, suggesting that regio-specific N-glycan distribution might occur already in non-malignant ovarian pathologies. All in all, our data provide proof that the combination of MALDI-MSI and sialic acid derivatization is suitable for delineating regio-specific N-glycan distribution in EOC and BOT tissues and might serve as a promising strategy for future glycosylation-based biomarker discovery studies.

Keywords: *in situ N*-glycosylation; sialylation; ovarian cancer; imaging; MALDI-MS

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Grzeski M, Tauber E, Braicu EI, Sehouli J, Blanchard V[#], Klein O[#] (2022). „In-situ N-glycosylation signatures of epithelial ovarian cancer tissue as defined by MALDI Mass Spectrometry Imaging". Cancers **14**(4):1021. [#] equally contributed.

A3) Neu5Ac and Neu5,9Ac$_2$ in Human Plasma: Potential Biomarkers of Cardiovascular Disease

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Cardiovascular disease (CVD) poses an extreme global healthcare burden, accounting for 32% of all deaths in 2019. CVD is characterized by increased levels of inflammation which has been associated with a rise in the concentration of *N*-acetyl neuraminic acid (Neu5Ac) in blood. The ability for the marker to distinguish between disease cases and healthy controls was not clear, however. Neu5Ac is just one of a large, diverse family of neuraminic acids. 9-*O*-Acetyl-*N*-acetyl-neuraminic acid (Neu5,9Ac$_2$) has been of interest as a biomarker for diseases such as breast cancer but has not been studied in the case of CVD. Neu5Ac and Neu5,9Ac$_2$ concentrations were determined by quantitative analysis using ultra-high-performance liquid chromatography in plasma samples obtained from both patients with CVD and healthy controls. Mean concentrations of Neu5Ac and Neu5,9Ac$_2$ were significantly elevated between the two sample groups (Neu5Ac: $P < 0.001$; Neu5,9Ac2: $P < 0.04$). Receiver operator curve analysis (ROC) further revealed the predictive power of the two markers (Neu5Ac AUC: 0.95; Neu5,9Ac2 AUC: 0.84). A combined Neu5Ac/Neu5,9Ac$_2$ marker exhibited an AUC of 0.96. The sensitivity (true positive) and specificity (true negative) of each marker was subsequently assessed. Neu5Ac and Neu5,9Ac$_2$ both showed good specificity (Neu5Ac: 0.9; Neu5,9Ac$_2$: 0.9), but only Neu5Ac showed adequate sensitivity (Neu5Ac: 0.88; Neu5,9Ac$_2$: 0.5). The combined Neu5Ac/Neu5,9Ac$_2$ marker offered similar sensitivity (0.88) to Neu5Ac but a much higher specificity (1.0) indicating a zero false positive rate. Overall, Neu5Ac appears to have good discriminatory power for CVD. Combining the two markers together may offer a better biomarker than either of the markers individually. Further analysis was undertaken to determine the N-glycan profile and percentage galatosylation of N-glycans. ROC analysis was performed and AUC values were calculated for these analytes and compared with the ROC data for Neu5Ac and Neu5,9Ac$_2$. The samples were also analysed via nanoparticle prefractionation. This allowed for the extraction of fibrinogen, which is a known inflammatory marker, from the plasma and subsequent analysis of fibrinogen derived glycans. Following this, analysis was performed to determine any increase in the concentrations of these glycans between CVD cases and healthy controls.

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A4) Cell-line model of immunoglobulin G glycosylation uncovers RUNX1 as a potential modifier of the immunoglobulin G glycan profile

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Down syndrome (DS) is a condition caused by trisomy 21 that entails numerous symptoms, one of which are premature signs of aging. As revealed by our recent research, these signs of premature aging are also reflected in plasma-derived immunoglobulin G (IgG) glycosylation, which is a well-known marker of biological age (Krištić et al. 2014). We have recently uncovered that individuals with DS show on average a 19-year increase in biological age when compared to chronologically age-matched controls of normal karyotype, however the mechanism that causes this substantial difference is yet to be discovered. We hypothesize that these differences could be explained by the presence of a third copy of certain chromosome 21 genes, followed by the increased expression of proteins encoded by those genes. One candidate chromosome 21 gene is *RUNX1* based on a recent GWAS study which found SNPs around this gene to be associated with human plasma IgG glycosylation (Klarić et al. 2020), and its extra copy has been discovered as an initiator of leukemogenic predisposition in DS (Nižetić and Groet 2012). We here used EBV-immortalized lymphoblastoid cell lines (LCLs) from an individual with DS and their disomic parent and sibling and treated the cells with a chemical inhibitor of the protein encoded by *RUNX1*. After treatment, the comparison of glycosylation profiles of IgG generated by these LCL cells revealed that inhibition of RUNX1 very significantly affected the glycosylation of IgG from LCLs derived from the disomic controls, yet no significant change in profile was observed in the cell-derived IgG of the person with DS. This finding complies with the aforementioned GWAS results and further implies that RUNX1 could be an important modulator of the general IgG profile in people with a normal number of chromosomes. Other mechanisms may prevail in skewing the glycan profiles in cells from people with DS.

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A5) Effect of bisection on the 3D structure and recognition of N-glycans during maturation with implications of the expression of MGAT3 in biological function

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Abstract:

N-glycosylation is a highly conserved post-translational modification in eukaryotic cells. N-glycosylation relies on highly coordinated, complex cellular machinery operating through the endoplasmic reticulum (ER) and the Golgi, ultimately translocating the glycosylated protein through the secretory pathway. The diversity of N-glycans is cell-dependent and their heterogeneity relies heavily on the expression of linkage and/or monosaccharide-specific glycoside hydrolases (GHs) and glycoside transferases (GTs), responsible for branching and functionalisation. In this work we are interested in the modification carried out by the N-Acetylglucosaminyltransferase-III, also known as MGAT3 from the encoding gene, which is responsible for linking a GlcNAc in b4 of the central mannose on an N-glycan pentasaccharide core, also called bisecting GlcNAc. This modification has been reported by some research groups to inhibit the functionalization of the N-glycans arms and overexpression of MGAT3 has been found to be linked to cancer and only common in specific tissues, such as brain and kidney. Meanwhile there is irrefutable evidence of bisection occurring in the context of fully functionalized biantennary N-glycans. In this work, we explore the structural architecture of these bisected N-glycans comparatively with fully functionalised tri- and biantennary N-glycans using molecular dynamics (MD) simulations. We use this information to characterise their potential recognition by two GTs involved in the maturation of N-glycans, namely beta-1,4-Galactosyltransferase 1 (b4GAlT1) and alpha-(1,6)-fucosyltransferase (FUT8), to gain insight into 'if and how' bisection hinders the maturation of N-glycans with implications of the expression of MGAT3 with the N-glycans' glycocode.

A7) Functional analysis of isoforms of CMP-sialic acid transporter

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The CMP-Sia transporter (CST) is a member of the Solute Carrier family SLC35, highly mammalian conserved type III trans-membrane antiporter (Nji et al., 2019; Zhao et al., 2006). Due to the importance of the presence of Sia in glycoconjugates, its transporter has been investigated and recently the mouse CST (mCST) has been crystallized, which has helped to determine its functionality. However, some splice variants from CST continue without being characterized. One of these is the del177 isoform, which loses the exon 6 and in humans is still functional, unlike other reported isoforms like the isoform del130, del124 or del290. (Ahuja & Whorton, 2019; Martinez-Duncker et al., 2005; Salinas-Marín et al., 2016). However, the del177 isoform in other mammals had already been reported to be nonfunctional like in the Lec2 cells that's derived from of a mutant of the Chinese hamster ovary cell line (CHO) (Eckhardt et al., 1998, 1999;). With the aim to propose an explanation about ability to transport CMP-sialic for del177 variant, we assess the effect of the c.303 C>G (p. Gln101His) missense mutation previously reported for alter 50% of CMP-Sialic acid transporter (Mohamed et al., 2013; Riemersma et al., 2015) in the isoform del177 through transfections in Hek293t knockout (KO) cells. Where SIA transport was measured by staining with Sambucus Nigra Lectin (SNA) and Peanut Agglutinin Lectin (PNA), in addition to a proliferation assay with RCA II lectin to observe SIA deficiency in transfected wild type (wt) and KO cells.

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A8) *C*-Mannosylation of Semaphorins

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C-Mannosylation is a type of protein glycosylation in which a single mannose is bound via a C-C bond to tryptophan residues that are commonly part of the consensus sequence WxxW/C. The enzyme catalyzing this type of glycosylation called *C*-mannosyltransferase was initially identified in *C. elegans* to be the gene product of *dpy-19* (Büttner et al, 2013). In mammals four homologues of DPY-19, designated as DPY19L1 to L4 are present, of which DPY19L1 and DPY19L3 have been proven to be active *C*-mannosyltransferases (Shcherbakova et al, 2017). Target proteins for *C*-mannosylation comprise thrombospondin type one repeat (TSR) domain-containing proteins, e.g. the *C. elegans* protein MIG-21 (Büttner et al, 2013) and its mammalian homologue SEMA5 (John et al, 2021). Both proteins have been shown to be involved in neuronal migration but the importance of *C*-mannosylation for their function is not known (Middelkoop et al, 2012; Adams et al, 1996).
SEMA5A and SEMA5B contain multiple TSRs and in order to map and quantitatively determine the degree of *C*-mannosylation, fragments comprising different TSRs of these proteins were recombinantly expressed in wild-type, DPY19L1- and DPY19L3-deficient CHO cells and analyzed by mass spectrometry. This analysis showed that several tryptophan residues belonging to different TSRs of SEMA5A and SEMA5B are *C*-mannosylated in CHO wild-type cells. Upon recombinant expression of these constructs in DPY19L1- or DPY19L3-deficient CHO cell lines, *C*-mannosylation of distinct tryptophan residues was affected. We quantitatively determined the degree of *C*-mannosylation of target tryptophan residues upon recombinant expression of different SEMA5A and 5B constructs containing different numbers of TSRs. Thereby we intend to study a potential competition of tryptophans belonging to different TSRs for *C*-mannosylation. We further deleted the *C*-mannosyltransferases DPY19L1 and/or DPY19L3 in human induced pluripotent stem cells (hiPSC) by CRISPR-Cas9 and differentiated the obtained knockout cells into neurons endogenously expressing SEMA5A. Initial experiments suggest that SEMA5A is not localized at its target location at the plasma membrane in DPY19L1-deficient hiPSC-derived neurons.

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A9) Action of multivalent galectins on FGFs fine-tunes cellular signaling and determines cell fate

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Fibroblast growth factors (FGFs) family includes eighteen secreted proteins that employ fibroblast growth factor receptors 1-4 (FGFR1-4) in transmitting signals through the plasma membrane (Ornitz et al, 2022). Balanced FGFs signaling is critical for human development and health. and dysregulated FGF/FGFR signaling is observed in about 8% of human cancers (Helsten et al, 2016). Secreted FGFs are organized into six subfamilies: FGF1 (FGF1 and FGF2), FGF4 (FGF4, FGF5, FGF6), FGF7 (FGF3, FGF7, FGF10, FGF22), FGF8 (FGF8, FGF17, FGF18), FGF9 (FGF9, FGF16, FGF20) and FGF19 (FGF19, FGF21, FGF23). Among these groups only FGF1 family members are devoid of classical signal sequence and are exported from the cell in an unconventional manner (La Venuta et al, 2015). Vast majority of FGFs (16 proteins) include putative N-glycosylation sites and pass ER/Golgi compartments during their secretory pathway. However, the presence of N-glycans on secreted FGFs and the functional significance of these modification are largely unknown.
Here we produced in mammalian cells representative members of all five groups of secreted FGFs as fusions with the Fc fragment of IgG1: FGF4-Fc, FGF9-Fc, FGF10-Fc, FGF18-Fc and FGF23-Fc, provided evidence for their glycosylation and tested the significance of N-glycosylation for FGFs stability, FGFR binding and activation. In the extracellular space secreted N-glycosylated FGFs may experience abundant galectins, β-galactoside-binding lectins capable of N-glycans recognition, strongly implicated in cancer (Johannes et al, 2018). Using biochemical and biophysical approaches, we screened for galectins forming complexes with FGFs in N-glycan dependent manner. We identified galectin-1, -3, -7 and -8 as novel binding partners of secreted FGFs. Using biolayer interferometry (BLI) we provided evidence for a direct and N-glycosylation-dependent sub-micromolar affinity interaction between model FGFs and identified galectins. Our preliminary data indicate that, depending on galectin type, binding of galectins to N-glycans of FGFs differentially affect FGF interaction with heparin, an essential FGF/FGFR signaling complex component. Importantly, N-glycan-dependent binding of FGFs to galectins modifies the ability of FGFs to activate FGFRs and FGFR-dependent downstream intracellular signaling pathways. Our data decipher novel mode of FGF/FGFR signaling regulation, where information stored as N-glycans within FGFs is read by extracellular galectins and used to fine-tune cellular signaling.

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A10) Structure and function of the accessory secretion system in gut symbionts

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The accessory secretion (aSec) system is a protein export pathway uniquely present in gram-positive bacteria and dedicated to the secretion of large, glycosylated cell-wall anchored adhesins called serine rich repeat proteins (SRRPs). It has been primarily characterised in pathogens in the context of biofilm formation and virulence. It is comprised of the secretion machinery (SecA2, SecY2, Asp4), chaperones (Asp1, Asp2, Asp3) and a variable number of glycosyltransferases (GTs) that *O*-glycosylate the secretory target. Recent discovery of aSec and SRRPs in *Limosilactobacillus reuteri* strains ATCC 53608 and 100-23 provided new insights into the structure, strain specific glycosylation and function of SRRPs in symbiotic gut bacteria (Latousakis et al, 2018; Latousakis et al, 2019).

Here a combination of biochemical and structural approaches were used to investigate the structure and function of aSec components *L. reuteri*. ATCC 53608 and 100-23 strains. Recombinant proteins of the *L. reuteri* aSec pathway were produced in *E. coli* and purified individually or in complex.

The crystal structure of *Lr*GtfC$_{100\text{-}23}$, one of the GTs involved in strain-specific glycosylation of SRRP (Latousakis et al, 2019), was determined by X-ray crystallography showing a classical GT-B fold. Site-directed mutagenesis of *Lr*GtfC$_{100\text{-}23}$ revealed the importance of Ser238 in conferring UDP-Glc specificity as shown using thermal shift assays while *Lr*GtfC$_{53608}$ C240W mutation introduced promiscuity with positive thermal shifts for both UDP-Glc and UDP-GlcNAc ligands.

A combination of small-angle X-ray scattering (SAXS) and Alphafold2 were used to construct structural models of individual aSec components and complexes. The *Lr*SecA2Y2Asp4$_{53608}$ complex consisted of a predicted dimerised motor-ATPase SecA2, membrane protein SecY2 and Asp4. *Lr*Asp1-2-3$_{53608}$ formed a 1:1:1 complex, where *Lr*Asp2$_{53608}$ exhibited acetylesterase activity. Binding of native SRRPs purified from both ATCC 53608 and 100-23 showed high binding avidity to both *Lr*Asp1-2-3$_{53608}$ and the secretion complex *Lr*SecA2Y2Asp4$_{53608}$ (Kd = 10^{-7}-10^{-8} M) with cross-strain reactivity.

Together these data suggest a highly regulated and conserved secretion process that progresses the SRRP secretion cargo through *O*-glycosylation, *O*-acetylation, and transport in a sequential manner. This work opens new avenues for glycoengineering applications by transferring the aSec system into heterologous hosts for the secretion of glycosylated proteins.

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A11) High-throughput N-glycosylation alpha-1-acid glycoprotein analysis could aid in the diagnosis of diabetes mellitus

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Alpha-1-acid glycoprotein (AGP) is an acute phase glycoprotein in blood, which is primarily synthetized in the liver. Although many possible functions of AGP have been described, the detailed knowledge about its exact role in pathophysiological processes is still elusive. A large part of AGP (around 45%) is modified by glycans, so they certainly play a very important role in its biological activity. Recently, we have developed a cost-effective method for a high-throughput and site-specific N-glycosylation LC-MS analysis of alpha-1-acid glycoprotein (AGP) which can be applied on large cohorts, aid in search for novel disease biomarkers, and enable better understanding of AGP's role and function in health and disease (Keser et al, 2021). The method does not require isolation of AGP with antibodies and affinity chromatography, but AGP is enriched by acid precipitation from 5 µl of blood plasma in a 96-well format. After trypsinization, AGP glycopeptides are purified using a hydrophilic interaction chromatography-based solid-phase extraction and analyzed by reversed-phase-liquid chromatography-electrospray ionization-MS. We used our method to show for the first time that AGP N-glycan profile is stable in healthy individuals, but it is variable between the individuals of the same population. Furthermore, we tested our method on a population including individuals with registered hyperglycemia in critical illness (59 cases and 49 controls), which represents a significantly increased risk of developing type 2 diabetes. Individuals at higher risk of diabetes presented increased N-glycan branching on AGP's second glycosylation site and lower sialylation of N-glycans on AGP's third and AGP1's fourth glycosylation site. We also analyzed fucosylation of AGP in subjects with HNF1A-MODY (most common type of monogenic diabetes in adults) and other types of diabetes aiming to evaluate its diagnostic potential in 564 individuals (Tijardović et al, 2022). The results showed significant reduction in AGP fucosylation associated to HNF1A-MODY when compared to other diabetes subtypes. Additionally, ROC curve analysis confirmed significant discriminatory potential of individual fucosylated AGP glycopeptides, where the best performing glycopeptide had an AUC of 0.94 (95% CI 0.90–0.99). Altogether, our results indicate that AGP glycosylation could have a significant role in diagnosis and understanding of diabetes mellitus.

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A12) Glycan analysis of lung tissue: in relation with COVID-19 disease

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Coronavirus disease, also known as COVID-19, is spreading through our community from the end of the year 2019. The most damaged organ in our body is lung. In the most severe cases, patient can be at a risk of a cytokine storm. When we focus on histopathological changes of lung tissue, the main finding is diffuse alveolar damage (Angeles Montero-Fernandez and Pardo-Garcia 2021). Glycan analysis showed higher α-2,6-sialylation in COVID sera and lower lobe lung tissues, but the amount of tissue samples was relatively small (4 controls and 4 COVID samples in duplicates) (Qin, Kurz et al. 2022).
In our study, we focused on optimization of effective protein extraction from lung tissue fixed in 70% ethanol, exchange of extraction buffer for more appropriate one with methods of lectin-based microarray and mass spectrometry and optimization of these glycoanalytical methods. We were working with lung tissue samples from 10 controls that died without positive test for SARS-CoV-2, 11 samples of patients that succumbed to the COVID-19 disease and 5 samples of patients that succumbed with COVID-19 disease.
We were able to prepare glycoprofiles of these samples by lectin-based microarray and mass spectrometry. We found trends in sialylation and fucosylation by lectin-based microarray and recorded profiles of N-glycans by mass spectrometry. We also tried technique of lectin blotting where we obtained increasing trend of α-1,2-fucosylation in a group of samples from patients, that succumbed to the COVID-19 disease.

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A13) Structural basis of glycan recognition by oligosaccharyltransferase

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Oligosaccharyltransferase (OST) is an integral membrane complex located at the membrane of the endoplasmic reticulum. It catalyzes the central reaction of *N*-linked protein glycosylation, the transfer of the pre-assembled oligosaccharide $GlcNAc_2$-Man_9-Glc_3 from a dolichyl-pyrophosphate (Dol-PP) carrier to asparagine residues in secretory proteins. Attachment of the complete glycan is required for efficient glycoprotein folding and intracellular trafficking. However, the molecular basis of the precise glycan recognition by OST remains unknown. Here we present cryo-EM structures of *S. cerevisiae* OST in distinct functional states bound to the chemo-enzymatically produced lipid-linked oligosaccharide (LLO) Dol20-PP-$GlcNAc_2$-Man_9-Glc_3. We found that the LLO glycan is recognized at two distinct regions representing the initiating and the terminating steps of LLO biosynthesis. At the reducing end, the $GlcNAc_2$ moiety is recognized by the catalytic subunit STT3, whereas the terminal glucoses (Glc3) at the non-reducing end are accommodated in a binding pocket formed by the non-catalytic subunits WBP1 and OST2. Furthermore, we found that binding of either donor or acceptor substrate alone leads to the formation of primed states of OST, and only the subsequent binding of the other substrate triggers the conformational changes in the catalytic subunit STT3 required for catalysis.

A14) Structural and functional characterization of yeast ALG10, a GT-C glucosyltransferase involved in eukaryotic protein N-glycosylation

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N-linked glycosylation is an essential protein modification that affects protein folding, stability and activity (Varki et al, 2017). At the endoplasmic reticulum of eukaryotes, a set of asparagine-linked glycosylation (ALG) enzymes use activated sugars to synthesize a lipid-linked oligosaccharide (LLO), which acts as glycan donor in the oligosaccharyltransferase-catalyzed reaction (Schwarz et al, 2011). The last enzyme in the LLO biosynthesis pathway is ALG10, an integral membrane protein belonging to the C superfamily of glycosyltransferases (GT-Cs) (Albuquerque-Wendt et al, 2019; Burda et al, 1998). It catalyzes the addition of the terminal α-1,2 glucose to the A-branch of the LLO. This final step is required for efficient N-glycosylation, as loss-of-function mutations of the enzyme result in severe hypoglycosylation phenotypes in yeast (Burda et al, 1998). In humans, ALG10 has been implicated in diseases such as colorectal cancer (Xu et al, 2022). At present, the catalytic mechanism and the molecular basis of donor and acceptor substrates recognition by ALG10 are not understood. Here, aided by synthetic Fab fragments, we report a first high-resolution cryo-electron microscopy structure of ALG10. The structure suggests possible substrate binding sites and allowed us to identify catalytic residues. We validated our structural observations by *in vitro* activity assays using an acceptor substrate analog (Dol25-PP-$GlcNAc_2Man_9Glc_2$), which we prepared using a chemo-enzymatic approach. Our results broaden our mechanistic understanding of eukaryotic N-glycosylation and foster the development of glycoconjugate therapeutics *in vitro*.

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A15) Glycation leads to increased invasion of Glioblastoma cell lines

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Glioblastomas (GBM) arise from astrocytes and are the most common and aggressive brain tumour. GBM cells disseminate and infiltrate the surrounding brain parenchyma rapidly, making complete surgical removal difficult. GBM cells are known to reprogram their metabolism to aerobic glycolysis (Warburg effect), leading to increased production of the by-product Methylglyoxal (MGO), a potent inducer of dicarboxylic stress. MGO has pro-tumorigenic characteristics and has been shown to lead to increased poly-sialylation. Poly-sialylation, which results from overexpression of sialyltransferases, is seen as a hallmark of cancer as it enhances immune evasion, tumour cell survival and stimulates invasion and migration.
In this study, we investigated the influence of MGO on the expression of sialyltransferases, on poly-sialylation and invasion. The cell lines LN229, U251 and human astrocytes (hA) were incubated without and with different concentration of MGO. Cell viability was measured using XTT assays, and cell morphology was depicted by microscopy. Protein glycation and poly-sialylation was analysed by Western blotting. The *Real Time Cell Analyzer (xCelligence)* was used to measure invasion and adhesion. The expression of sialyltransferases was examined on the mRNA level using quantitative polymerase chain reaction (qPCR).
MGO showed cytotoxic effects at 1 mM MGO, but cells remained alive up to a concentration of 0.3 mM in all cell lines. Glycation was increased in a concentration-dependent manner in all cell types after incubation with MGO. Invasion was increased by 50% in the two GBM cell lines LN229 und U251 but decreased in the hA by 20 %. The sialyltransferases expression showed great variation between the different cell lines with some sialyltransferases (ST6GAL2 and ST8 SIA3) only being expressed by the hA. The sialyltransferases responsible for poly-sialylation (ST8SIA2 and ST8SIA4) were upregulated in the LN229 and hA after glycation but downregulated in the U251. A general increase in the expression of all sialyltransferases could only be seen in LN229. This could result in poly-sialylation accompanied by increased invasion. Our study represents the first demonstration that MGO could act as a tumour-promoting factor in GBM cells. This opens the field for new potential diagnostic biomarkers and therapeutic strategies such as MGO scavengers and Glyoxalase1 activators.

A16) A Broad-Specificity O-Glycoprotease Enables Improved Analysis of Glycoproteins and Glycopeptides Containing Intact Complex O-Glycans

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Protein glycosylation (N- and O-glycosylation) plays an important role in the stability, activity and pharmacokinetics of many therapeutic glycoproteins *in vivo*. While current glycomic and glycoproteomic methods allow for comprehensive analysis of N-glycan structures and mapping of N-glycan attachment sites, analysis of mucin-type O-glycans linked to serine/threonine of glycoproteins remains technically challenging due to natural features of O-glycosylation and a dearth of enzymatic tools that enable their analysis. Ultimately, analytical methods that enable to map O-glycosites and profile a protein's O-glycan structures in a single facile experiment would be ideal. Recently several O-glycan-specific endoproteases that can generate O-glycopeptides have been described. This class of enzymes has great potential in glycoproteomic workflows as they can generate glycopeptides with an O-glycan residing at a defined position (*e.g.*, the N-terminal amino acid). However, existing commercial enzymes do not efficiently generate glycopeptides bearing sialylated complex O-glycans. Sialic acid typically needs to be enzymatically removed prior to its use, significantly limiting the richness of O-glycan structural data that can be obtained.

In this study, we describe the specificity of an O-Glycoprotease (immunomodulating metalloprotease (IMPa) from *Pseudomonas aeruginosa*) that cleaves proteins N-terminally to O-glycosites bearing common mucin-type O-glycans, including complex sialylated glycans and glycans comprised of a single GalNAc. In this poster, we summarize key findings from the recent study (Vainauskas et al, 2022) and illustrate the use of O-Glycoprotease for mapping O-glycosites and glycan profiling of the highly complex mucin-type glycoprotein CD45.

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A17) Structural and mechanistic basis of capsule *O*-acetylation in *Neisseria meningitidis* serogroup A

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Neisseria meningitidis serogroup A (*Nm*A) has been the major cause of bacterial meningitis in the African meningitis belt for decades. A major breakthrough in combatting the disease worldwide has been the development of glycoconjugate vaccines consisting of the capsule polymer of the pathogen coupled to a carrier protein. The capsule of *Nm*A consists of *O*-acetylated $[\rightarrow 6)$-α-D-ManNAc-$(1\rightarrow OPO_3^-\rightarrow]_n$ repeating units, and *O*-acetylation of the N-acetylmannosamine (ManNAc) residues at *O*-3 and to a minor extent at *O*-4 was found to be mandatory to induce a sufficient immune response after vaccination. The enzyme catalyzing this modification, CsaC, belongs to a so far structurally uncharacterized family of *O*-acetyltransferases and represents an important synthetic tool for vaccine developmental studies. Using comprehensive NMR studies, we demonstrate that *O*-acetylation catalyzed by CsaC stabilizes the labile anomeric phosphodiester-linkages of the *Nm*A capsule and occurs in position C3 and C4 of the ManNAc repeating unit due to enzymatic transfer and non-enzymatic ester migration, respectively. To shed light on the enzymatic transfer mechanism, we solved the crystal structure of CsaC in its apo and acceptor-bound form and of the CsaC-H228A mutant as trapped acetyl-enzyme adduct in complex with CoA. Together with the results of a comprehensive mutagenesis study, the reported structures explain the strict regioselectivity of CsaC and provide insight into the catalytic mechanism, which relies on an unexpected Gln-extension of a classical Ser-His-Asp triad, embedded in an α/β-hydrolase fold.

Reference

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A18) Glycosylation-dependent clustering modulate FGFR signaling and cell fate by a network of extracellular galectins

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At the cell surface fibroblast growth factor receptors (FGFRs) and their canonical ligands fibroblast growth factors (FGFs) form signaling platforms that regulate critical cellular processes, like cell division, motility, differentiation, metabolism and death (Ornitz et al, 2022). FGFRs signaling is critical for human body development and homeostasis and aberrant FGFRs are found in several diseases, including diverse cancer types. FGFRs are N-glycosylated at several positions, however the significance of these modifications is still mysterious (Duchesne et al, 2006).
Galectins constitute a family of twelve soluble, secreted lectins in human (galectin-1, -2, -3, -4, -7, -8, -9, -10, -12, -13, -14, and -16), implicated in a plethora of cellular processes, including signaling, cell proliferation, motility, endocytosis, autophagy and apoptosis, and in various diseases including cancers (Johannes et al, 2018). Galectins are capable of reading glyco-code in form of N-glycosylation of cell surface proteins (including RTKs) and transforming it into particular cellular effect (Porębska et al, 2021). In cancer, galectins promote cell division and survival, and facilitate evasion of cancer cells from the immune system (Girotti et al, 2020). Using mass spectrometry (MS)-based screening approach we have recently linked FGFRs and galectins by defining galectin-1 and galectin-3 as novel FGFR1 activators and regulators (Kucińska et al, 2019). We have shown that the glyco-code within FGFR1 is read by galectin-1, resulting in activation of the receptor, triggering cell division and avoidance of apoptosis, whereas galectin-3 affects endocytosis of the receptor. Since different galectin family members represent large variability in the oligomeric state and substrate specificity, potentially leading to distinct cellular effects on particular cellular N-glycosylated target, we decided to study in detail an interplay between all human galectins and FGFRs. We have produced all human galectins and used these proteins together with recombinant FGFRs to screen for galectins capable of FGFRs binding using biochemical approaches and biolayer interferometry (BLI). We identified galectin-7 and galectin-8 as novel FGFR1 interactors. Using several FGFR truncated variants and BLI we have shown that galectins bind with sub-micromolar affinity to N-glycans present in the FGF binding domains D2 and D3 of FGFRs. We have shown that while galectin-3/-7/-8 compete for the same binding sites on FGFRs, only galectin-7 and galectin-8 are capable of FGFR activation. Our data point on tight functional interdependence between FGFRs and galectins, which might be especially pronounced in cancer.
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A19) *N*-glycan sulfation on human IgA: Glycoproteomic in-depth analyses of sulfated *N*-glycan site-specificity and distribution on human IgA isotypes

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Human Immunoglobulin A plays a key role in immune response modulation in serum and, as secretory form, in pathogen neutralization on mucosal surfaces. Specific *N*-glycosylation sites influence neutralization and effector functions. While *O*-glycosylation on the hinge region influences IgA's potency, *N*-glycosylation on the IgA Fc region enhances neutralization activity and the complement activation function (Maurer et al. 2018). Especially, *N*-glycosylation on the C_H3 domain is reported as a target for viral hemagglutinins. Interestingly, hemagglutinin variants have affinity for different glycoforms e.g., for sulfation (Maurer et al. 2018; Stevens et al. 2006). Previous research of our group on *N*-glycans released from human IgA has identified one sulfated *N*-glycan moiety via xCGE-LIF. Chuzel *et al* confirmed the sulfation linked to the carbon 6 of antenna GlcNAc of the *N*-glycan moiety (Chuzel, L., *et al 2021*). However, the protein *N*-glycosylation site(s) harboring sulfated *N*-glycans remained unclear. Here, *N*-glycosylation of human IgA was investigated using a mass spectrometric workflow with focus on evidence of sulfated *N*-glycan moieties and their individual glycosylation sites. Glycosylation sites N340 in IgA1, N205 in IgA2 and N144 in IgA1/ N131 in IgA2 were identified as the origin of all sulfated *N*-glycans in human IgA. A software-assisted manual validation and subsequent relative quantification on pure IgA subunits confirmed N340 in IgA1 with 3.07% sulfated *N*-glycopeptides. After analyzing unfractionated human IgA from two different manufacturers, sulfated *N*-glycans were consistently observed in N340 IgA1, confirming that this is the primary site of sulfated *N*-glycosylation. Nevertheless, sulfated *N*-glycopeptides represent a minor share of 0.08% of the total human IgA peptides. Furthermore, evidence for other seven mono- and di-sulfated *N*-glycan moieties was collected. These findings have so far not been described in previous research. Secretory IgA is a possible target to enhance the protective mucosal responses, whereas serum IgA is an interesting target for developing therapeutic IgA monoclonal antibodies in cancer treatment (Neutra and Kozlowski 2006; Sousa-Pereira and Woof 2019). Therefore, the various immunologic functions of IgA are not only promising for medical research, but also for enhancing its potential as therapeutic.

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A20) Branched *N*-glycans of colonic stromal cells as key players during colitis-associated colorectal carcinogenesis

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Inflammatory Bowel Disease (IBD)-associated Colorectal Cancer (CAC) is a major concern in the clinical management of patients with chronic Inflammatory Bowel Disease (IBD) (Choi et al, 2017). Inflammation is a driving factor both in IBD and cancer, but the mechanism underlying the progression to CAC remains poorly understood. Dysregulation of mucosal glycosylation has been described as a key regulatory mechanism associated both with colon inflammation and colorectal cancer (CRC) development (Leite-Gomes et al, 2022). Our group showed that decreased expression of *MGAT5* glycogene on intestinal T lymphocytes from IBD patients was associated with disease severity (Dias et al, 2014; Dias et al, 2018). On the other hand, we also demonstrated that the aberrant expression of *MGAT5* glycogene in CRC was associated with immune escape (Silva et al, 2020). However, whether this differential expression of glycans in colitis and cancer contexts mediated by *MGAT5* glycogene constitutes a new mechanism in CAC remains completely unknown. A well-characterized cohort of CAC patients (from Porto University Centre Hospital – Portugal and Hospital Clinico San Carlos – Madrid, Spain) at different stages of carcinogenesis (colitis, dysplasia and cancer) was assessed to evaluate the *N*-glycosylation profile of stroma cells. Furthermore, in vivo studies (using AOM/DSS to induce CAC) were conducted in *MGAT5* WT and KO mice. The glycoprofile and immunoprofile of colon T cells was analyzed. Our cross-sectional study showed a distinct glycoprofile in the stroma, characterized by an increase of branched N-glycans along the carcinogenic cascade, with an increased expression of *MGAT5* gene in high-risk colitis. Additionally, colonic T cells isolated from AOM/DSS *MGAT5* glycoengineered mice presented a switch on *N*-glycans composition along CAC development, followed by different inflammatory profiles, which showed an effect in tumor development. Our preliminary results demonstrate a distinct glyco-signature of colonic stromal cells along the colitis-associated carcinoma cascade, which appears to impact tumor development.

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A21) Why are Cys domains of mucins not C-mannosylated?

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Abstract:

Mucins are the major component of the mucus, the first biological barrier at the surface of secretory epithelia. Mucins are high molecular weight glycoproteins and they occur secreted or membrane-bound. Serine-, threonine- and proline-rich regions are highly O-glycosylated. These S/T/P regions are interrupted by Cys domains (CysDs), which are small domains with 8 conserved cysteine residues. CysDs contain conserved WxxW motifs, the consensus for C-mannosylation. Therefore, it has been hypothesized that CysDs are C-mannosylated. However, previous studies could not proof this. Hence, we investigated the potential C-mannosylation on mucin CysDs by mass spectrometry. We recombinantly expressed CysD5 of human MUC5AC in CHO cells. MUC5AC is a secreted, gel-forming mucin. We purified His-tagged CysD5 with Ni-NTA-chromatography and analyzed digested peptides by LC-MS. MS analysis revealed that this CysD is not C-mannosylated, even if the protein is retained to the ER.
However, we showed that CysD are C-mannosylated if conserved cysteine-residues are mutated. The mutated CysDs were almost fully C-mannosylated. These cysteine-residues form intramolecular disulfide bridges. We compared these results to the CysD from cartilage intermediate layer protein 1 (CILP1). This CysD contains the conserved WxxW motif but not the mentioned cysteine-residues. In CHO cells, CILP1 CysD is C-mannosylated.
Our experiments suggest that the motif of mucin CysDs is a potential substrate for C-mannosyltransferases, but cysteine-residues hinder C-mannosylation. We propose that formation of intramolecular disulfide bridges or co-translational binding of chaperones to adjacent cysteine-residues prevents C-mannosylation.

A22) Lacto-*N*-tetraosyl ceramide is a novel marker for human pluripotent stem cells

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Human pluripotent stem cell-derived somatic cells are envisioned to be applied in regenerative medicine in future. Thereby, the differentiated cells cannot contain any residual pluripotent stem cells, which might otherwise lead to teratoma formation in the human recipient by uncontrolled proliferation. The glycosphingolipid-based pluripotency markers SSEA3, SSEA4, and SSEA5 are commonly applied to characterize the pluripotent state of stem cells and can also be used for elimination of residual pluripotent stem cells from differentiated populations. Applying our novel analytical approach for profiling of glycosphingolipid glycosylation by multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF) [Rossdam *et al.*], we set out to reassess human pluripotent stem cell-specific glycosphingolipids. Thereby, we observed that levels of the glycosphingolipid-derived glycan lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Lc4) were considerably reduced upon differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) into early stages of the three germ layers mesoderm, endoderm, and ectoderm, suggesting the potential of Lc4 as a novel pluripotency marker. The decrease of Lc4 levels during the first four days of differentiation was confirmed by flow cytometry upon live cell surface staining using an Lc4-specific antibody. Using this antibody, we could additionally sort the cells into an Lc4-positive and Lc4-negative population by magnetic cell separation (MACS). We were able to show by flow cytometry that the Lc4-negative cells are still positive for SSEA3, SSEA4, and SSEA5 while qPCR-based analysis of relative expression levels of additional stem cells markers, *OCT4* and *NANOG*, already revealed a differentiated state. In contrast, expression of *OCT4* and *NANOG* was much higher in the Lc4-positive cell population. These findings validate Lc4 as a novel and promising marker for undifferentiated stem cells, even suggesting that this marker is more specific for pluripotency than SSEA3, SSEA4, and SSEA5.

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A23) Rutinosidase: Glycosidase with unprecedented glycosylation activities controlled by a substrate tunnel

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Keywords: Glycosidase, transglycosylation, substrate tunnel, glycosyl azide.

Rutinosidases (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosidases, EC 3.2.1.168, CAZy GH5) cleave the glycosidic bond between the rutinose and the aglycone. Rutinosidase from *A. niger* also exhibits β-D-glucopyranosidase activity, and thus can be considered as a β-glucosidase with broad substrate specificity. This enzyme employs rutin as a rutinosyl donor or quercetin-3-β-glucoside as a glucopyranosyl donor. The crystal structure revealed strong and specific binding of aromatic aglycon (quercetin) and high tolerance at the glycon binding site. This enzyme can glycosylate (both with rutinosyl and β-glucosyl) many substrates such as alcohols (primary, secondary, and tertiary), phenols, and aromatic acids (glycoside at carboxy groups). Non-aromatic glycosylation products are no longer enzymatically hydrolyzed. Even the wild type rutinosidase can glycosylate azide to form β-rutinosyl or β-glucosyl azide, which is a yet undescribed phenomenon in the nonmutated glycosidases. The active-site mutant E319A can generate α-rutinosyl (or α-glucosyl) azide (Kotik et al, 2021). The unique abilities of this enzyme are plausibly caused by a substrate tunnel in the structure of rutinosidase, which may explain the unusual catalytic properties of this glycosidase and its strong and unusual transglycosylation potential (Brodsky et al, 2020).

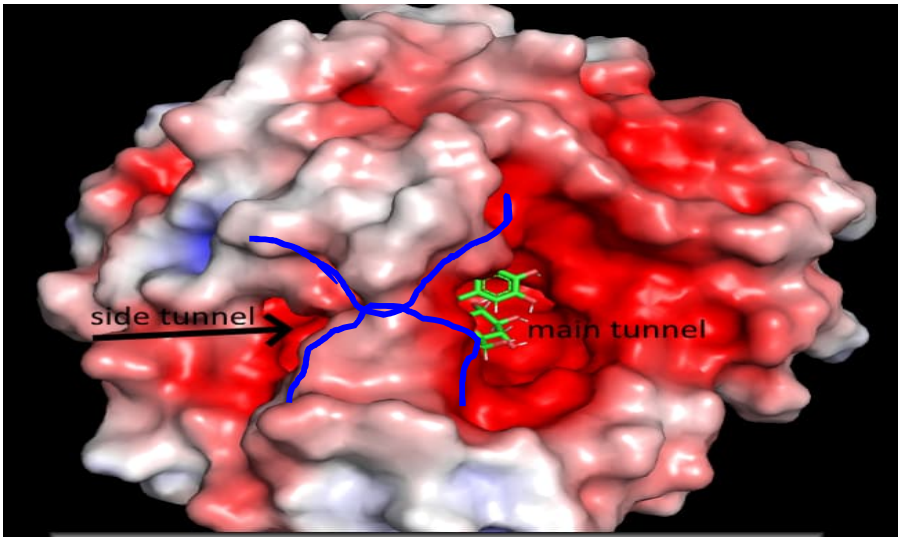

Figure 1. Substrate tunnels of rutinosidase from *A. niger* (rutin in the active site). The bridge over the active site building the side tunnel is formed by interactions of two loops (blue).

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Novel Emerging & Enabling Systems in Glycobiotechnology

B1) Development of a cell-free enzymatic cascade for the synthesis of GDP-fucose - modeling and optimization.

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Glycans fulfill essential biological roles central to human health. They can be found in their free form or conjugated to proteins and lipids. To increase their availability for both scientific and commercial use, there is growing demand to develop economical synthesis routes. The nucleotide sugar GDP-fucose is the substrate for the biosynthesis of fucosylated glycans. However, GDP-fucose is scarcely available, at prices exceeding 120 €/mg as its production through approaches like fermentation or chemical synthesis is expensive and elaborate.

As an alternative, we have established a scalable, cell-free enzymatic cascade reaction to synthetize GDP-fucose from inexpensive precursors, polyphosphate GMP and fucose (Fig. 1). The initial design of reactions and conditions was based on previous work by Mahour et al. To optimize the final product titer and to minimize the biocatalyst load enough to obtain a substrate conversion of at least 90%, a model based on Michaelis-Menten kinetics was established. The model was built using a set of reaction data for parameter estimation; the software COPASI was used as a platform for all simulation and optimization steps. Using this approach, the product titer could be increased from the initial value of 16 mM to 19.8 mM after 24 hours. In addition, the biocatalyst load was decreased from 0.04 mmol/L to 0.02 mmol/L with substrate conversion close to 99%.

Overall, this work demonstrates the potential of rationally designed modeling and optimization approaches to improve cell-free synthesis methods. This is paving the way for the commercial use of the technology. Through our spin-off eversyn® (www.eversyn.de), we are planning to provide low-cost nucleotide sugars and glycans for applications in the (bio-) pharma and nutrition industries in the near future.

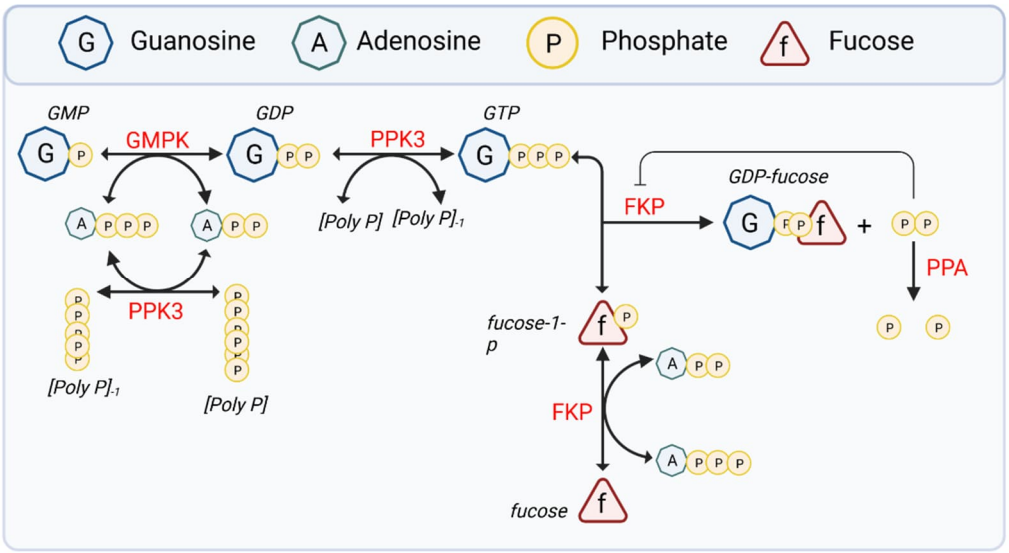

Figure 2 - Multi-enzyme cascade for the synthesis of GDP-fucose from fucose, polyphosphate and GMP.

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B2) Modular Economical One-Pot Multienzyme Synthesis of Complex Glycans

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Glycans are ubiquitous and play essential roles in diverse biological functions. Glycans have broad applications, including supplementation of human milk oligosaccharides to infant formula, design of glycan-based vaccines against pathogens and cancers, and functions of homogeneous glycoproteins, among many others. However, present synthesis methods fail in affordable scalability which hinders the development of glycan therapeutic applications. Even the wide-use FDA/EMA-approved pentasaccharide anticoagulant, fondaparinux, requires about 50 steps to be chemically synthesized, giving around 0.1% overall yield (Petitou et al, 1989). Current one-pot multienzyme (OPME) systems have streamlined numerous steps in glycan synthesis (Tsai et al, 2013); still, few have focused on their optimization and scalability, especially when integrating energy regeneration. In this work, we succeed in using a stable, affordable phosphate energy source to cycle different nucleotide sugars *in situ* for synthesizing complex glycans in one pot. Given the acidic and cation-sequestering nature of phosphate donors, large numbers of components in the reactions and their cross-interactions need to be assessed and optimized. We first enumerated all the possible factors and ranked their importance based on our understanding of the respective enzymology. Hard-to-change factors like different enzyme homologs have the lowest priority. We also established an efficient workflow to rapidly monitor the reactions at multiple time points by MALDI-TOF mass spectrometry; an in-house Python script was written to analyze and visualize countless mass spectra automatedly. Additionally, methods using design of experiments were adopted to systematically optimize the combined reaction mixture. Here, we demonstrate our workflow with the extension of lactose with β1,3-GlcNAc starting from inexpensive precursors and catalytic amounts of nucleotides. We have expanded and modularized our economical OPME system (ecOPME) into different sugars and linkages. Reduced input of nucleotides, especially expensive CTP and GTP, use of high sugar precursor concentrations, nearly 100% completion of the reactions, and minimal downstream repetitive purifications pave the path to multi-gram scale synthesis of complex glycans in an ordinary lab setup. Facile accessibility to complex glycans can facilitate research on their functions and applications that improve human health.

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B3) Leveraging Fluorine Directed Sialylation in the Stereoselective Synthesis of the Meningitis Type C Epitope (*Neisseria Meningitidis* C Polysaccharide Capsule)

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Along with proteins and DNA, carbohydrates are among the three most common biopolymers in nature (Varki, 2017) Of the most common monosaccharide building blocks, **sialic acids** play an essential role in regulating interactions with lectins, receptors and enzymes due to their location at the glycan termini (Schauer, 2009). Prominent glycan mimetics in drug discovery, Tamiflu and Relenza, have proven effective treatments for influenza through neuraminidase inhibition (Ernst & Magnani, 2009).
Classical enzymatic synthesis of carbohydrates poses a number of challenges including poor yields and impurities (McCarthy et al, 2018), which can be overcome through chemical design. Fluorine provides a useful tool to influence the stereoselective outcome of a reaction (Hayashi et al, 2019). In this poster we present sialic acid as a target for the development of a **carbohydrate-based vaccine candidate** through chemical synthesis. In a 17-step route we synthesised an α-2,9-linked **fluorinated disialoside**, a mimetic of the natural capsular polysaccharide epitope of the bacteria *Neisseria Meningitidis* (Pizza & Rappuoli, 2015).

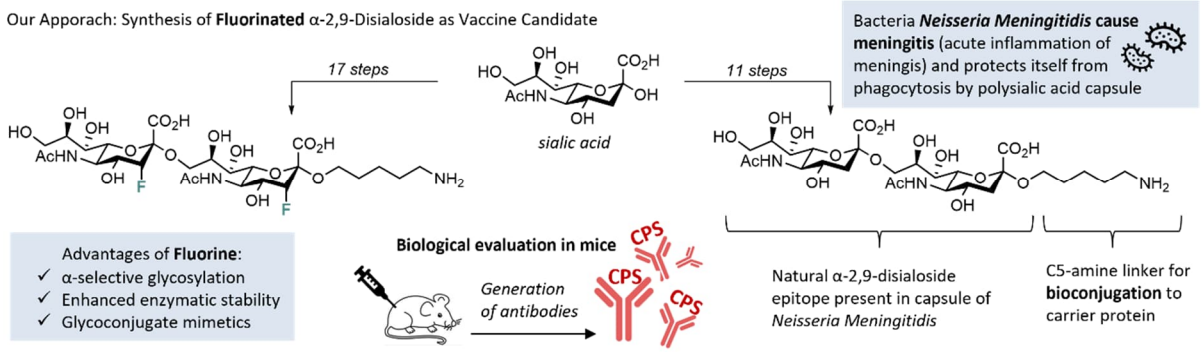

Figure 3: Starting from commercially available sialic acid (middle top), two carbohydrate-based antigens were targeted. The non-natural fluorinated disialoside (left) was synthesised α-selectively in 17 steps, whereas the natural disialoside (right) was afforded in 11 steps using a literature known procedure (Chu et al, 2011). Biological evaluation in mice show generation of capsular polysaccharide (CPS) specific antibodies. Comparison of both disialosides in glycan arrays were carried out. [unpub. results]

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B4) Towards the development of a semi-synthetic glycan based vaccine against *Neisseria meningitidis*

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Neisseria meningitidis is a gram-negative bacterium that causes meningitis. Capsular polysaccharides (CPS) are a major virulence factor and can be used as vaccine candidates, as they induce protective immune responses. Various types of serotypes, classified by the CPS structures, are found in patients where A, B and C are the most frequent ones (Rouphael and Stephens, 2012). A well-defined fluorinated disialoside, an oligosaccharide resembling the CPS of serotype C, was synthetized and was conjugated to different carrier proteins to test their suitability as vaccines in a mouse model. Our aim is to compare the immunogenicity and the raised protection of the produced antibodies using different carrier proteins and adjuvants. The first carrier protein is Porin A, which is produced by serotype B. Therefore, when the glycan is conjugated to Porin A, the vaccine can target two serotypes at once. Alternatively, the other protein is CRM197 because it is common in many human vaccines and it is known to enhance the immune response. Two adjuvant formulations are used: Alum, which is already approved for human vaccines, and Freunds Complete Adjuvant, widely employed in experimental mouse models. In fact, after screening the mice sera from different time points by glycan array and ELISA, we could observe that the vaccines induce an immune response. As well, the increase of the antibody production can be seen over time. Moreover, the antibodies recognize and bind to the whole bacteria and to the native CPS. In summary, we have shown that our vaccines trigger the production of specific antibodies and their protective efficiency is being investigated.

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B5) Autocatalytic Production of Polysialic Acid by Transient Expression in *N. benthamiana*

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Human polysialic acid (polySia) is the homopolymeric glycan of the nonulsonic sugar 5-N-acetyl neuraminic acid, a sialic acid derivative. It occurs as a tightly controlled posttranslational modification synthesized by the two polysialyltransferases (polySTs) ST8SiaII and ST8SiaIV on only few proteins, most notably in the human brain on the Neural Cell Adhesion Molecule during brain development. Besides its well-documented role in promoting neural plasticity, it recently has also been characterized as an immunomodulatory glycan (Karlstetter et al, 2017; Thiesler et al, 2021). Consequently, there is increasing demand for free polySia of ultra-high purity and of defined length. Currently, commercially available polySia originates from the capsule of the neuropathogenic bacterium *E. coli* K1 (Rode et al, 2008) in a production route that requires their large-scale fermentation, introducing certain drawbacks into the final product, among them pyrogen contamination.
In the past, we demonstrated the *in planta* functionality of human ST8SiaII and ST8SiaIV by transient expression in a *Nicotiana benthamiana* glycosylation mutant. To produce mammalian, complex-type core glycans that can act as the substrate for ST8SiaII and ST8SiaIV, a mammalian enzyme cascade was co-expressed (Kallolimath et al, 2016). By exploiting the autocatalytic and synergistic nature of the two polySTs, we sought to establish a scalable process of producing polySia in an environmentally friendly system, remote from both bacteria or mammals, which makes our established *N. benthamiana* platform a prime target. Polysialylated polySTs were produced by transient expression in *N. benthamiana* through agro-infiltration and isolated by polySia-specific immunoprecipitation. Conditions of infiltration, harvest and extraction maximizing polySia yield as well as purification-recovery were thoroughly investigated and applied. After release of the homopolymer from the residual glycoantenna, it can be directly purified by anionic exchange chromatography, arriving at a lyophilized end product after dialysis.

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B6) Synthesis of chemical reporters for chemoenzymatic and metabolic labelling of *O*-GlcNAcylated proteins during neural cell development.

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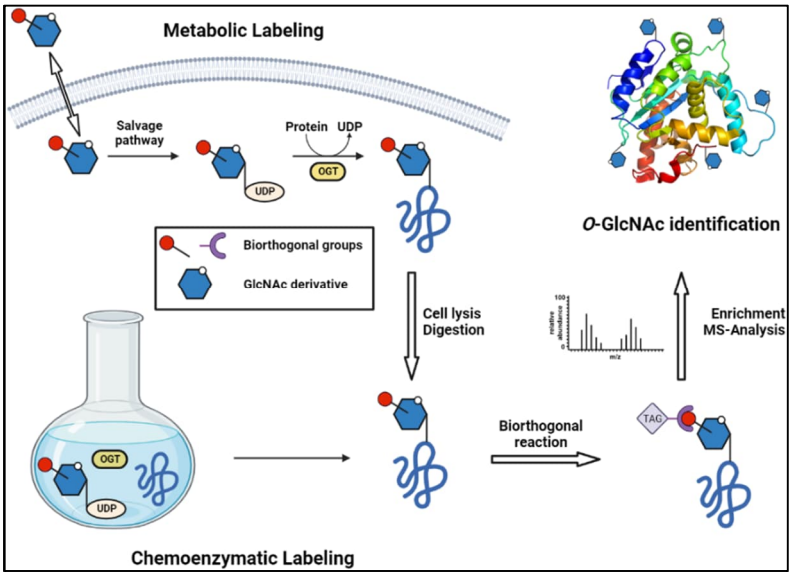
Glycosylation of Ser and Thr residues by N-acetylglucosamine (*O*-GlcNAc) is an important regulatory modification that controls essential biological processes of proteins. As aberrant *O*-GlcNAcylation patterns are associated with various chronic diseases such as diabetes, cancer, and neurodegeneration, knowledge of the precise positioning, stoichiometry, and dynamic changes of *O*-GlcNAc residues is vital to understand molecular protein (dys)functions. The predominant strategy for identifying and visualizing *O*-GlcNAcylation of proteins involves the labelling with chemically modified carbohydrate probes. Syntheses of novel and existing metabolic and chemoenzymatic reporters have been developed for *in vivo* and *in vitro* studies (Pedowitz et al, 2021; Wen et al, 2018).

These new and fully characterized labels have been employed for preliminary quantitative proteomic analyses. To map dynamic changes in the O-GlcNAcylome of developing neural stem cells (NSCs) similar studies will be pursued at distinct points in time during their differentiation by using our recently developed chemical proteomic SP2E protocol (Becker et al, 2022).



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B7) Development and characterization of SpyCatcher-Glycosyltransferase fusion proteins for immobilization in automated glycan synthesis

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Enzymatic synthesis of complex glycans is an established approach in glycobiotechnology (Rexer et al. 2021). Obstacles to the use of Leloir glycosyltransferases (GTs, EC 2.4.1), such as the availability of nucleotide sugars, have been overcome reaching almost industrial scale (Fischöder et al. 2019; Frohnmeyer et al. 2022).
Automation of enzymatic synthesis has been addressed, most often as solid-phase synthesis with immobilized acceptor substrates or as catch-and-release strategies (Wen et al. 2018). However, in many processes, the GTs are denatured or otherwise discharged after the reaction, leading to unnecessarily high costs. Therefore, a versatile, cost-efficient, and automated process with high reusability of GTs is needed.
Our project “MiRAGE” aims at the development of a counter-current flow reactor with immobilized GTs for highly selective automated glycan synthesis. GTs are fused with the SpyCatcher peptide (SpyC) from *Streptococcus pyogenes* (Keeble et al. 2019) for covalent immobilization in a microgel network via SpyCatcher/SpyTag self-assembly. We here present a set of different GTs that were equipped with SpyC. All constructs are actively expressed in *Escherichia coli* strains. We hereby show that the fusion of SpyC to the catalytic domains of the GTs has no negative influence on their activity. We aim to produce eight different SpyC-GTs and determine their optimal reaction conditions. Furthermore, the immobilization and combination of these enzymes to functional cascades are evaluated and integrated into an automated versatile process for enzymatic glycan synthesis.

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B8) Transport Phenomena in Automated Glycan Assembly (AGA)

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Glycans are the most abundant biomolecules on earth, from cellulose in biomass to host glycans in the spike protein of SARS-CoV-2 (Zhao et al, 2021), understanding their structure/function relationships is crucial for several biological applications. Automated Glycan Assembly (AGA) allowed access to glycans in the order of 100mer with high coupling yields and stereoselectivity (Joseph et al, 2020). When compared to oligonucleotides and peptide synthesis (SPPS), synthesizing the relatively more complex structure of glycans requests more flexible process conditions, such as extreme temperatures variations (from -40 to 90 °C), alternating the reaction solvents and acidity, using large donor excess and high coupling times (Danglad-Flores et al, 2021). Regarding process time, a full-coupling cycle in SPPS might take up to 2 min, for AGA is thirty times higher (Joseph et al, 2020; Danglad-Flores et al, 2021; Seeberger et al, 2015). The ever-need to optimize the system, increase productivity and facilitate access to pure glycans, demands the investigation of the process from a non-synthetic point of view. Therefore, a quantitative study of the transport phenomena and physicochemical processes in AGA is paramount. We found that the coupling step is the major time contributor with 30% of the total AGA cycle. We theorize that particle swelling, intraparticle diffusion, and coupling to the solid support need to be described in order to minimize the general cycle time.

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B9) Mix-and-Match System for the Enzymatic Synthesis of Enantiopure Capsule Polymer Backbones from *Actinobacillus pleuropneumoniae*

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Capsule polymers are crucial virulence factors of pathogenic bacteria and are used as antigens in glycoconjugate vaccine formulations. Some Gram-negative pathogens express poly(glycosylglycerol phosphate) capsule polymers that resemble Gram-positive wall teichoic acids and are synthesized by TagF-like capsule polymerases. So far, the biotechnological use of these enzymes for vaccine developmental studies was restricted by the unavailability of enantiopure CDP-glycerol, one of the donor substrates required for polymer assembly. Here, we use CTP:glycerol-phosphate cytidylyltransferases (GCT) and TagF-like polymerases to synthesize the poly(glycosylglycerol phosphate) capsule polymer backbones of the porcine pathogens *Actinobacillus pleuropneumoniae* (*App*) serotypes 3 and 7. GCT activity was confirmed by high-performance liquid chromatography and polymers were analyzed using comprehensive NMR studies. Solid-phase synthesis protocols were established to allow potential scale-up of polymer production. Additionally, one-pot reactions exploiting glycerol-kinase allowed to start the reaction from inexpensive, widely available substrates. Finally, this study highlights that multi-domain TagF-like polymerases can be transformed by mutagenesis of active site residues into single-action transferases, which in turn can act in *trans* to build-up structurally new polymers. Overall, our protocols provide enantiopure, nature-identical capsule polymer backbones from *App*2, *App*3, *App*7, *App*9 and *App*11.
This study was funded by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - project number 412824531.

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B10) MULTIFUNCTIONAL CHIRAL HYDROGEN AND HALOGEN BOND CONTAINING CATALYSTS IN GLYCOSYLATION REACTIONS

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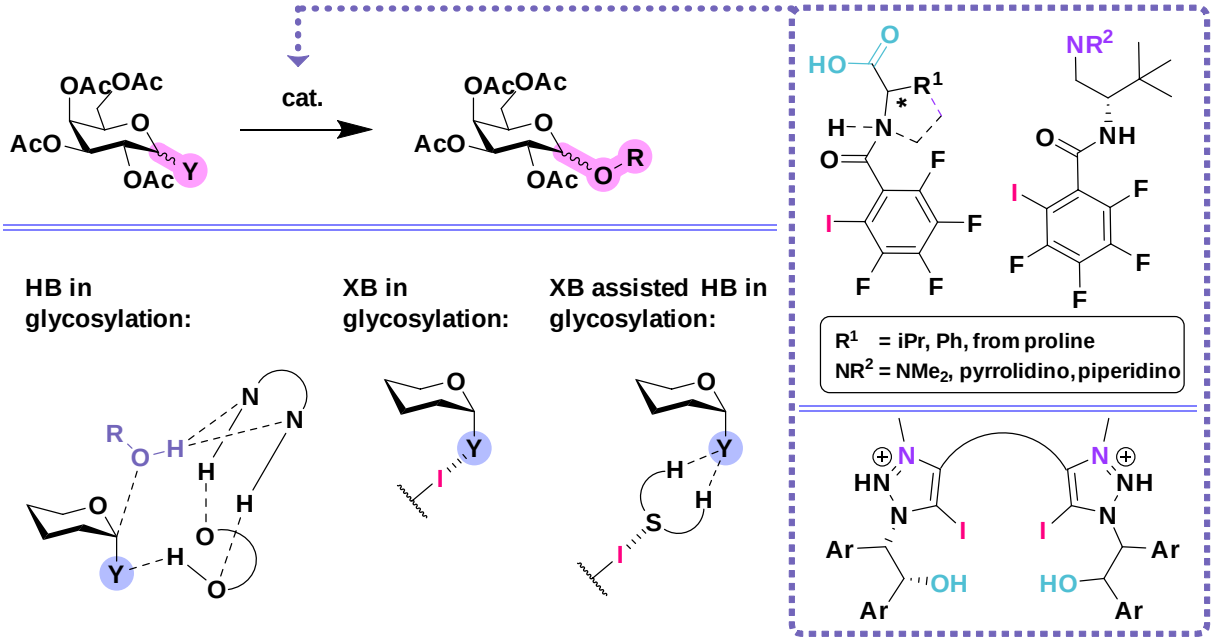
Glycosylation reactions have a 140-year history with many of the original procedures for saccharide synthesis still in use today (Nielsen et al, 2018). Although acid catalysis, hydrogen bond catalysis and organocatalysis are all well-established methods for catalytic glycosylation reactions, halogen bond (XB) catalysis has yet to have been systematically adopted for this type of reaction, outside of a few singular examples (Gallier et al, 2022). Here, a wide range of chiral bifunctional XB-catalysts and organocatalysts were applied to a glycosylation reaction with the aim of attaining anomerically pure sugars. Different donor groups were utilized and the significance of the role of the halogen bond in the formation of the product sugars was assessed.



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B11) Development of an *in-vitro* multi-enzyme cascade for the synthesis of uridine diphosphate *N*-acetylgalactosamine using a DoE approach

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Biocatalytic production of active pharmaceutical ingredients, nutritional components and other valuable molecules is becoming increasingly important. In general, either cell-free or whole cell synthesis can be utilized. Both approaches exhibit several advantages and disadvantages (Tao et al., 2011). For instance, whole cell biocatalysts provide inherent co-factor regeneration, whereas in cell-free system undesired side reactions can be avoided (Straathof et al., 2000). Here, a cell-free multi-enzyme cascade producing the nucleotide sugar uridine diphosphate *N*-acetylgalactosamine (UDP-GalNAc) was developed. This activated molecule is an essential element of various glycoproteins and glycolipids in human milk that modulate the immune response of infants (Figueroa-Lozano et al., 2019). To date UDP-GalNAc is commercially only available in mg quantities with prices exceeding 70 €/mg.

The cascade established consists of six recombinant enzymes overexpressed in *E. coli* (Mahour et al., 2018; 2022). In one-pot batch reactions, the inexpensive substrates uridine, polyphosphate and GalNAc are converted to UDP-GalNAc. To reduce costs, ATP is *in situ* regenerated from polyphosphate. Starting at 30 °C, pH 7.5 and 45 mM $MgCl_2$ and a batch time of 24 h, a conversion yield of approximately 10 % and a final product titer of 2.9 g/L could be obtained from initial substrate concentrations of 50 mM uridine and GalNAc, respectively.

By employing a statistical design of experiments (DoE) approach, we aimed at increasing the product yield through optimization of the process parameters temperature, pH and $MgCl_2$ concentration. Therefore, an experimental range of 30-40 °C, pH 7-9 and 45-100 mM $MgCl_2$ was screened. By using a D-optimal design, a set of 21 experiments was conducted. The optimal conditions identified were 39 °C, a pH of 8.9 and 96.5 mM $MgCl_2$. Using these parameters, the yield could be increased to 80 % with a final UDP-GalNAc concentration of 26.8 g/L.

Through our spin-off eversyn® (www.eversyn.de) we will provide nucleotide sugars and glycans in large scales for applications in the (bio-)pharma and the nutrition industry in the near future.

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B12) Multi-Gram-scale production of uridine nucleotide sugars via *in-vitro* multi-enzyme cascades

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Large-scale enzymatic synthesis of functional oligosaccharides and glycoconjugates such as human milk oligosaccharides and glycoproteins is still limited by the price and availability of their building blocks, nucleotide sugars (Faijes et al., 2019; Figueroa-Lozano et al., 2019). We have successfully developed an *in-vitro* multi-enzyme cascade consisting of six recombinant enzymes to produce the nucleotide sugars UDP-galactose (UDP-Gal) and UDP-*N*-Acetylglucosamine (UDP-GlcNAc) from the inexpensive precursors UMP, polyphosphate, and GlcNAc and galactose, respectively (Mahour et al., 2018; 2022). For cost reduction, the relatively expensive co-substrate ATP is *in situ* regenerated from polyphosphate. Moreover, utilizing a set of duet vectors, the entire cascade is overexpressed in one single *E. coli* strain.

Using a protein lysate as biocatalyst, UDP-GlcNAc is produced with a conversion yield of 100 % with respect to 100 mM UMP in a batch process at 100 mL scale within 24 hours. The final product concentration was 61 g/L. Using the same set-up, UDP-Gal can be produced with a conversion yield of 90 % and a final product titer of 51 g/L. The high conversion yield allowed applying ultrafiltration with a cut-off of 10 kDa for product purification leaving polyphosphate as the major impurity for UDP-GlcNAc and UDP-Gal, respectively.

To further improve product purity, a scalable anion exchange chromatography protocol was established using HiTrap Q HP columns from Cytiva – a strong anion exchange resin. With a dynamic binding capacity of approximately 24 $mg_{nucleotide}/mL_{resin}$, milligram-scale purification per run utilizing mL-scale columns is possible.

Through our spin-off eversyn® (www.eversyn.de) we will provide nucleotide sugars and glycans in large scales for applications in the (bio-)pharma and the nutrition industry in the near future.

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B13) Enzyme cascades for the synthesis of nucleotide sugars: Updates to recent production strategies

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Nucleotide sugars play an essential role in nature and serve as natural substrates for Leloir glycosyltransferases. However, effective production systems for nucleotide sugars are spare or poorly discussed. We discuss developing new approaches to nucleotide sugar synthesis and production strategies based on critical production parameters such as the space-time yield (STY), the total turnover number (TTN), and product yields. Valuable nucleotide sugars such as UDP-Gal, UDP-GalNAc, and UDP-GlcNAc were produced in the multi-gram scale by applying the repetitive-batch (rep-batch) procedure (Fischöder et al. 2019). Extraordinarily high TTNs of 494 g_P/g_E (UDP-Gal), 522 g_P/g_E (UDP-GlcNAc), and 398 g_P/g_E (UDP-GalNAc) were obtained for a 5-day production week (Fischöder et al., 2019). Economic access to UDP-GlcA, UDP-GlcNAc, and GDP-Fuc was developed by integrating an ATP regeneration system with polyphosphate kinase (PPK) and polyphosphate (polyP), leading to high conversion yields (Gottschalk et al. 2021 and Frohnmeyer et al. 2022). We demonstrated the synthesis of up to 1.6 g of GDP-Fuc in rep-batch mode with a final TTN of 27 g_P/g_E. However, a higher TTN of 31 g_P/g_E was reached with ATP excess. The latter synthetic approach converted up to 25% of the substrate to ADP-Fuc as site product. GDP-Fuc was purified by sequential steps of phosphate precipitation, where the nucleotide sugar was treated with an alkaline phosphatase solution in the first step, and >95% of orthophosphates were precipitated at -20 °C by supplementing the solution with 1.5 Volumes of 2-propanol and GDP-Fuc afterward precipitated by supplementing the solution by adding 2 volumes of 2-propanol. This protocol recovered 77% of GDP-Fuc (Frohnmeyer et al. 2022).
UDP-GlcA and UDP-GlcNAc were efficiently synthesized by enzyme cascade immobilization on magnetic beads (Gottschalk et al., 2022). Multiple uses of the magnetic beads up to the fifth cycle resulted in high average STYs of 4.3 $g*L^{-1}*h^{-1}$ for UDP-GlcA and 6.3 $g*L^{-1}*h^{-1}$ for UDP-GlcNAc (Gottschalk et al. 2022).

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B14) Protective capacity of a recombinant vaccine against the cattle parasite *Ostertagia ostertagi* relies on engineering its native N-glycan composition

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With the rising resistance against anthelmintic drugs, a more sustainable control strategy against parasitic nematode infections in livestock, such as vaccination, is required. Vaccination with native activation-associated protein 1 from the economically important cattle parasite *Ostertagia ostertagi* (Oo-ASP-1) have demonstrated to induce protection (Meyvis et al 2007). However, collecting large quantities of Oo-ASP-1 is difficult as it requires inoculation and sacrifice of large numbers of animals, which is labour-intensive, time-consuming and ultimately unsustainable for large-scale production. For this reason, vaccine development is dependent on recombinant expression of this protein. Attempts have been made to express Oo-ASP-1 in, for instance, yeast and baculovirus expression systems, but failed to confer the same level of protective capacity as native Oo-ASP1 due to difference in protein folding and post-translational modifications (Geldhof et al 2008; González-Hernández et al 2016). Here, we show that glyco-engineering native glycan structures on recombinant Oo-ASP-1 expressed in *Nicotiana benthamiana* restores the protective capacity of the vaccine. Recombinant Oo-ASP-1 is easily expressed in high quantities in *N. benthamiana* and subsequently purified without complex downstream processing. Furthermore, the N-glycosylation of native Oo-ASP-1 is mimicked by co-expression of specific glycosyltransferases. In vaccination trials, calves vaccinated with recombinant Oo-ASP-1 demonstrated a strong reduction in faecal egg counts and a significant increase in local IgG1 and IgG2 antibody responses, which correlates with protective immunity. These results indicate that mimicking the native N-glycan composition of Oo-ASP-1 in *N. benthamiana* is required for its vaccine efficacy. Furthermore, these results demonstrate that N-glycosylation is an essential component for developing effective recombinant vaccines against parasitic nematodes.

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B15 + B16) ALiCE®: High-yielding, scalable, eukaryotic cell-free protein expression for rapid production of N-glycoproteins

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Eukaryotic cell-free protein synthesis (CFPS) systems have the potential to simplify and speed up the expression and high-throughput analysis of complex proteins with functionally relevant post-translational modifications (PTMs). However, low yields and the inability to scale such systems have so far prevented their widespread adoption in protein research and manufacturing. Here, we present a detailed demonstration for the capabilities of ALiCE®, a CFPS system derived from *Nicotiana tabacum* BY-2 cell culture. Owing to this eukaryotic origin, ALiCE retains native organelle machinery in the form of 'microsomes', hybrid reformations of endoplasmic reticulum and Golgi components. The system is therefore able to express diverse, functional proteins at high yields in 48 hours, complete with disulfide bonds and N-glycosylation.

Recent advances in the scaling of BYL production methodologies have allowed scaling of the CFPS reaction and we show simple, linear scale-up of batch mode reporter protein expression from a 100 µL microtiter plate format to 10 mL and 100 mL volumes in standard Erlenmeyer flasks. Scaling of reactions yields greater amounts of protein products for more detailed characterisation. Thus, we present N-glycan analyses from a panel of interesting model proteins, specifically: a dimeric enzyme, glucose oxidase; the monoclonal antibody adalimumab and the SARS-Cov-2 receptor-binding domain. Functional binding and activity are shown using a combination of surface plasmon resonance and a serology-based ELISA method. Finally, in-depth PTM characterisation of purified proteins revealed the correct formation of intra- and intermolecular disulfide bonds, with homogenous, high occupancy N-glycan profiles typical of plant-derived glycoproteins i.e. high mannose structures with some plant-specific α1,3-fucosylation and β1,2-xylosylation.

Taken together, BYL provides a real opportunity for screening of complex proteins at the microscale with subsequent amplification to manufacturing-ready levels using off-the-shelf protocols. This end-to-end platform suggests the potential to significantly reduce cost and the time-to-market for high value proteins and biologics.

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Das Gupta, M., et al. (2022). "ALiCE®: A versatile, high yielding and scalable eukaryotic cell-free protein synthesis (CFPS) system." [Manuscript submitted for publication]

B17) Resent developments in Solid Phase Automated Glycan Assembly

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Automated glycan assembly (AGA) is significantly more challenging compare other to linear biomolecules, as highly branched carbohydrates require strict regio- and stereocontrol during synthesis. A new generation of AGA synthesizer explote the wider range of temperature from −40 to +100 °C to control glycosylations at low temperature and accelerates capping, protecting group removal, and glycan modifications using elevated temperatures. Thereby, the temporary protecting group portfolio is expanded from two to four orthogonal groups. In addition, sulfated glycans and unprotected glycans can be prepared. The new instrument drastically shortens and generalizes the synthesis of carbohydrates for use in biomedical and material science.

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B18) Development of a cell-free multi-enzyme cascade for the synthesis of CDP-glycerol

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CDP-glycerol is the nucleotide-activated form of glycerol. In Gram-positive bacteria, it is one of the substrates for the biosynthesis of teichoic acid, which is an appealing target epitope for the development of new vaccines. Here, we report on the development of a cell-free multi-enzyme cascade to synthetize nucleotide-activated glycerol from the inexpensive and readily available substrates cytidine and glycerol. The cascade consists of six recombinant enzymes that are expressed in *E. coli* and purified by immobilized metal affinity chromatography. As part of the cascade, ATP is *in-situ* regenerated from polyphosphate to reduce synthesis costs. The enzymatic cascade was tested at lab-scale and the reaction products analyzed by high-performance anion-exchange chromatography (HPAEC)-UV and MALDI-MS. In a first trial, the successful synthesis of CDP-glycerol was confirmed. The substrate conversion yield was 10% and the final product titer was 1 mM after a batch time of 18 hours. A design of experiments approach was used to screen for optimal operation conditions (temperature, pH value and $MgCl_2$ concentration). For this purpose, a fractional factorial design was selected and 17 cascade reaction runs performed in biological triplicates. The optimal conditions identified were a pH of 8.7, a temperature of 29.2°C and a MgCl2 concentration of 58.52 mM. Through increasing the substrate load and using the optimal reaction conditions, the substrate conversion yield was increased to 89% with respect to cytidine, and a final product titer of 31 mM was achieved after a batch time of 24 hours.

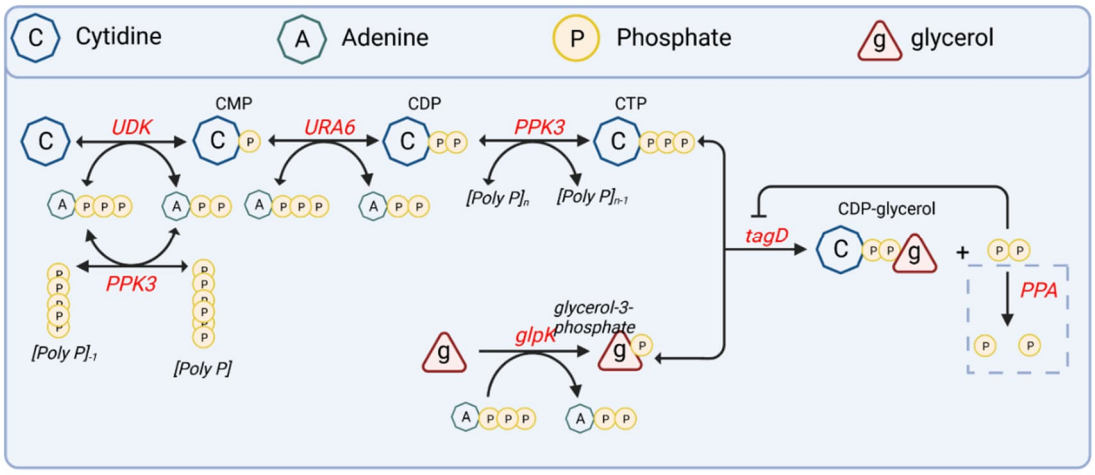

Figure 4- Multi-enzyme cascade for the synthesis of CDP-glycerol from cytidine, glycerol and polyphosphate

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B19) Development of a cell-free multi-enzyme cascade for the synthesis of CMP-Neu5Ac

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Cytidine 5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) is the nucleotide-activated form of *N*-acetylneruramic acid (Neu5Ac) and the substrate for the sialylation of glycoproteins by sialyltransferases (Makino et al., 1993). *N*-acetylneuraminic acid residues typically occur at the terminal position of the *N*-linked glycans. This monosaccharide hinders the recognition of proteins by hepatocytes, increasing its lifetime and making it an essential element in the production of therapeutic proteins (Ma et al., 2020). However, CMP-Neu5Ac is rather expensive.

Here, we report on the development of a cell-free multi-enzyme cascade to synthetize CMP-Neu5Ac from the inexpensive and readily available substrates CMP, pyruvate and *N*-acetylglucosamine (GlcNac). The cascade comprises six recombinant enzymes expressed in *E. coli.* As part of the cascade, ATP is *in-situ* regenerated from polyphosphate to reduce synthesis costs. The enzymatic cascade was implemented at laboratory-scale and the reaction substrates and products were analyzed by high-performance anion-exchange chromatography (HPAEC)-UV. In a first trial using purified enzymes and a substrate initial concentration of 10 mM, a conversion yield of 25% was obtained. After performing several experiments following a DoE screening strategy, optimal parameters and conditions were identified that resulted in a conversion yield of 91% after 8 h starting from 100 mM of CMP and using the lysate after the homogenization of the microorganism as the reaction medium to avoid purification costs.

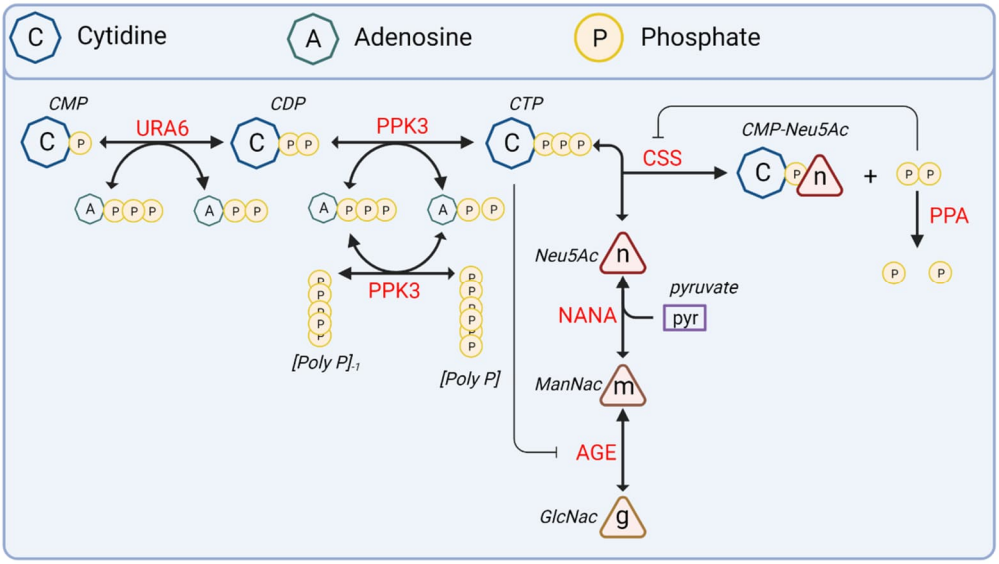

Figure 5- Multi-enzyme cascade for the synthesis of CMP-Neu5Ac from CMP, Pyruvate, GlcNac and polyphosphate

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Synthesis and Function of Human Milk Oligosaccharides

C1) Neo-glycoproteins with human milk oligosaccharides as high-affinity ligands of galectins

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Galectins are proteins that belong to the human lectin family. They are involved in biological and pathological processes such as cell signaling, cell adhesion, apoptosis, fibrosis, cancerogenesis, and metabolic disorders (Heine et al, 2022). Galectins consist of three types of structures: prototype, tandem-repeat, and chimeric. Tandem-repeat galectins contain two carbohydrate-recognition domains connected with a peptide chain. Their behavior has not been fully explored due to the lower stability of the galectin and its subunits. The binding of monovalent glycans to galectins is usually relatively weak. Therefore the presentation of carbohydrate ligands on multivalent scaffolds is efficient to increase the affinity of the glycoconjugate (Heine et al, 2021). The sequential application of the recombinant enzymes β4-galactosyltransferase, β3-galactosyltransferase, β3-*N*-acetylglucosamine-transferase, mutant β4-galactosyltransferase and β3-galactosynthase from *B. circulans* resulted in a library of human milk oligosaccharide (HMO) and poly-LacNAc ligands. They showed a high affinity to galectins (Gal-1, -3, -8, -9) in ELISA-type assays. These monovalent ligands were conjugated to human serum albumin to produce the multivalent glycoconjugates, neo-glycoproteins (Figure 1). The affinity to galectins of prepared neo-glycoproteins was in the nanomolar range.

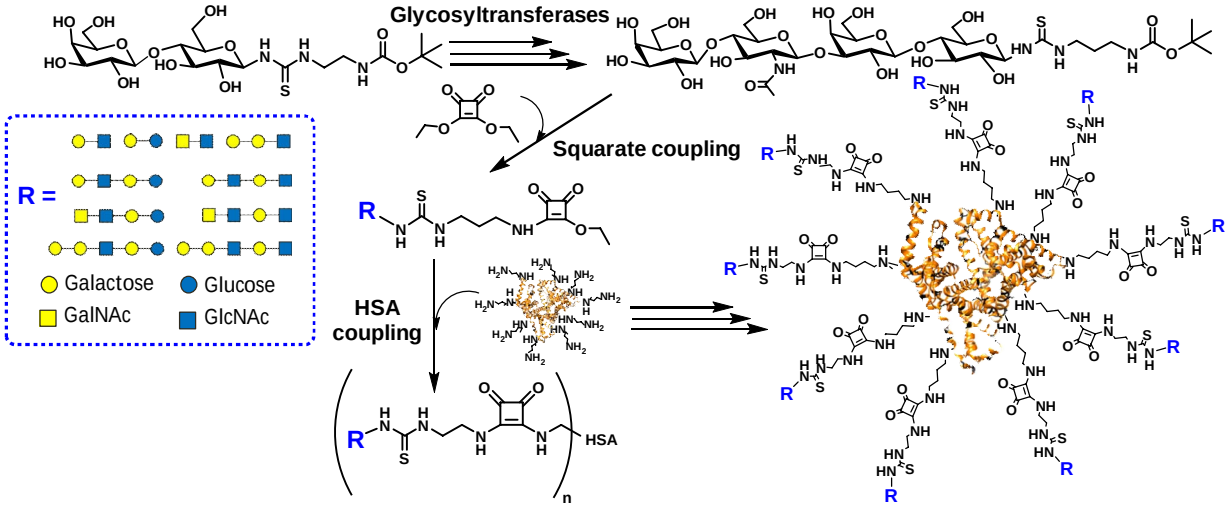

Figure 1. Chemo-enzymatic synthesis of human milk oligosaccharides (HMO) decorated neo-glycoproteins.

Support from the grant project 22-00317262S by the Czech Science Foundation is gratefully acknowledged.

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C2) Selective manipulation of acetyl groups in monosaccharides using *Candida antarctica* lipase-B for the synthesis of human milk oligosaccharides

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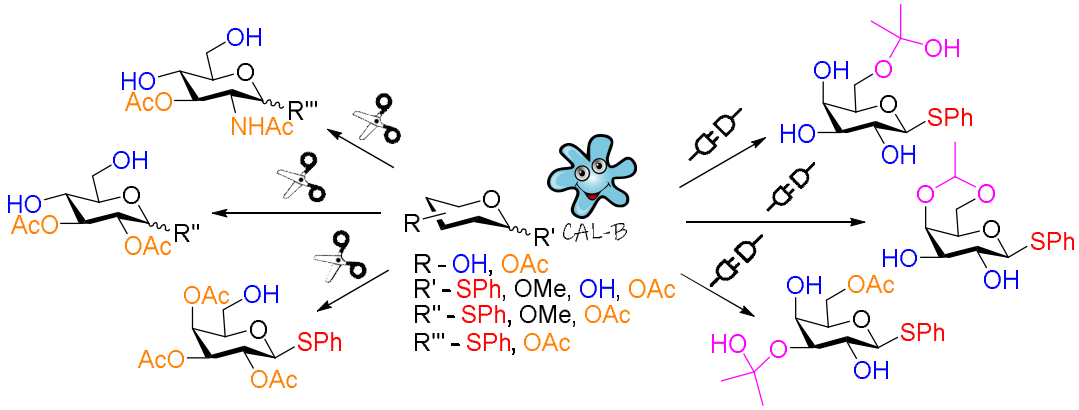
Human milk oligosaccharides (HMOs) are indigestible oligosaccharides only found in human milk. While HMO's main role is the development and growth of gut microbiota, they have been shown to have other beneficial attributes for the baby: act as decoys for pathogens, alleviate allergic symptoms etc. (Bode 2012). *Candida antarctica* lipase-B (CAL-B) has been used previously to selectively deprotect or acetylate various monosaccharides both in aqueous solutions and more importantly in organic media (Nicolosi et al. 2005, Riva et al. 1997).
Recently, we have selectively deacetylated four peracetylated HMO monosaccharide "building blocks" (D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc) and L-fucose (Fuc)) and their respective thioglycoside derivates with immobilized CAL-B in metyl tert-butyl ether and used two of the formed products to synthesize a deviant HMO – 6'-galactosyllactose. Some trends emerged in the deacetylation reactions: D-glucose based peracetylated saccharides (Glc, GlcNAc, D-lactose) and thioglycosides (Glc, GlcNAc) showed selectivity towards 4th and 6th position (only 6th for D-lactose). Peracetylated β-anomers (Glc, Gal) mostly preferred 1st position, and having the anomeric OAc group exchanged with either SPh or OMe slowed down the reaction significantly, except for GlcNAc with SPh leaving group (Kanger et al. 2022). Selective acetylation of monosaccharides depends heavily on the solvent and so far, several intriguing products have been acquired.



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C3) Efficient production of lactodifucotetraose (LDFT) using engineered *Escherichia coli*

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Human milk oligosaccharides (HMOs) have been suggested to have a variety of functions important for healthy infant development (intestinal environment, immunity, and brain function). Lactodifucotetraose (LDFT, Fucα1-2Galβ1-4(Fucα1-3)Glc) is a di-fucosylated tetrasaccharide which has both 2'-fucosyllactose (2FL, Fucα1-2Galβ1-4Glc) and 3-fucosyllactose (3FL, Galβ1-4(Fucα1-3)Glc) substructures. LDFT has been reported to be present in transition milk at about 0.5 g/L, and to have beneficial functions such as strong antibacterial activity against Group B *Streptococcus*. LDFT can be produced using engineered *Escherichia coli* co-expressing heterologous α1,2-fucosyltransferase (*α1,2-fucT*) and α1,3-fucosyltransferase (*α1,3-fucT*) genes, but the incomplete progression of the two-step fucosyltransfer reaction leads to the notable accumulation of intermediate 2FL or 3FL. A previous study attempted to produce LDFT in a single flow from lactose via 2FL by combining a lactose-specific α1,2-FucT (that cannot use 3FL as a substrate) and an α1,3-FucT that accepts 2FL as a substrate. In this study, we screened various combinations of α1,2-FucT and α1,3-FucT that can accept intermediate 2FL or 3FL as well as lactose and convert them to LDFT with high transfer activity. As a result, the combination of α1,2-FucT from *Helicobacter mustelae* and modified α1,3-FucT from *Bacteroides reticulotermitis* performed best. Furthermore, by adjusting the expression balance between α1,2-FucT and α1,3-FucT, a productivity of 73.5 g/L was achieved in a 3L jar fermenter culture (2FL 0.5 g/L and 3FL 0.7 g/L). The above culture medium was then purified by bacterial inactivation (acid and heat treatment), centrifugation, passage through ion exchange resin, membrane filtration, concentration, and crystallization to obtain a highly pure LDFT powder with a chromatogram purity of 98.6%. In the future, we will further improve the efficiency of LDFT synthesis, *i.e.*, the multi-step glycosylation reactions from lactose by optimizing the strain and culture conditions.

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C4) Identification of Dragon Fruit N-Glycans Released from Glycoproteins and Effects on Various Probiotic Microorganisms

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Dragon fruit belongs to the *Cactaceae* family from the genus *Hylocereus*. Production and consumption of fruit have recently increased globally. It plays critical roles in human health, including cancer chemoprevention, anti-inflammatory, and anti-diabetic actions. Studies show that dragon fruit is beneficial to human gut health due to its high oligosaccharide content. These glycans can be found in either free or conjugated form. When it is consumed by humans, glycans can get into the colon in an undigested form due to the lack of glycosidase enzymes in humans. Here, they can be utilized as a prebiotic carbon source by microorganisms present in the gut microbiota and stimulate selective growth. Studies show that dragon fruit oligosaccharides have a probiotic effect on some microorganisms. However, the bound glycan structures of the fruit are not clearly investigated. Due to research on conjugated glycans is rare, released N-glycans from dragon fruit may have a significant prebiotic influence on gut health. Increasing the number and activities of beneficial bacteria in the human gut microbiota compared to pathogens has great importance for human health, and at this point, the positive results of prebiotics on the microbiota draw attention.
In this study, research was conducted on the glycoproteins found in dragon fruit. Dragon fruit samples from two different locations were tested and visualized for protein content. To release N-glycans from the glycoproteins they attached, a unique glycosides enzyme was used. Characterization of novel N-glycan structures was analyzed through MALDI-TOF-MS. The pure glycans' prebiotic effect was evaluated using an in vitro digesting model.
As a result, we enlighten the information regarding the dragon fruit *N*-glycan profile and prebiotic activity studies of bioactive *N*-glycans. In this context, it has been explained how diverse glycoprotein-containing products affect the digestive system and shape the microbiome. Additionally, glycoprotein-based mechanisms on dragon fruit could help further study the complex nature of fruit-drug interactions and their potential impacts.

Acknowledgment:
The study was supported by The Scientific And Technological Research Council Of Turkey 2210-C Program and Çanakkale Onsekiz Mart University The Scientific Research Coordination Unit Project number: FYL-2021-3770.

Glycoengineering of Biopharmaceuticals

D1) Engineering human-like O-glycosylation in *Nicotiana tabacum* BY-2 cells

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Nicotiana tabacum BY-2 cell suspension cultures were demonstrated as a powerful platform for producing recombinant proteins. Because glycosylation is an essential feature of proteins, many efforts have been made to humanize the glycosylation profile of plant cells. However, scientific community has mostly focused on removal of non-human α(1,3)-fucose and β(1,2)-xylose *N*-glycans and, as a result, the *O*-glycosylation pathways are less understood and have still need to be addressed.

On the one hand, plant-specific *O*-glycans may be deleterious to the properties of recombinant protein and may be targeted by IgE (Leonard et al., 2005). The presence of these glycans has already been reported on several human glycoproteins such as EPO and IgA1 produced in plants (Parsons et al., 2013; Dicker et al., 2016). This glycosylation consists of hydroxylation of some proline residues by prolyl-4-hydroxylases (P4Hs) and subsequent addition of glycan moieties on the newly added oxygen atom. These glycans are separated into two classes, namely arabinose and arabinogalactan, which consist either of short arabinose chains or large galactose backbone modified with various side chains. We have identified 22 *P4H*-like genes in *N. tabacum*, which are clustered into six homology groups. To uncover which genes are involved in the aberrant hydroxylation of pharmaceutical glycoproteins, the genes from different groups will be knocked out with CRISPR/Cas9 technology. Using hyperglycosylated Ser-Pro repeats tags fused to GFP, we have designed a reporter protein that allows for a rapid screening of the knock-out mutants.

On the other hand, the typical mammalian mucin-type *O*-glycosylation pathway is absent in plant cells. This modification, which consists of glycan attachment to serine or threonine residues, is very important in many human proteins. In order to further humanize glycosylation, transgenic BY-2 cell lines expressing the enzymes responsible for the Core 1 and Tn antigen glycan formation were generated. The presence of these *O*-glycan structures was confirmed by lectin blotting. Several glycoproteins (EPO and IgA1) are currently being expressed in these engineered cell lines. Due to the absence of competition with endogenous enzymes, *O*-glycosylation is expected to be highly homogeneous.

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D2) Towards full control over protein glycosylation: *in-vitro* glycoengineering of influenza A virus hemagglutinin and SARS-CoV-2 spike protein

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The baculovirus-insect cell expression system is readily utilized to produce viral glycoproteins for research and pharmaceutical applications. This includes, for instance, subunit vaccines and vaccine candidates against Influenza and SARS-CoV-2 infections. However, with mainly complex-type *N*-glycans attached, the glycoforms of recombinant proteins derived from this expression system are inherently different from mammalian cell-derived glycoforms and the impact of these differences on the immunogenicity has hardly been studied in detail (Palomares *et al*., 2021; Schön *et al*., 2021). This applies also to the Influenza A virus hemagglutinin (HA) and the SARS-CoV-2 spike protein, which are antigen targets of nearly all licensed vaccines and vaccine candidates including virus like particles and subunit vaccines.
To generate specific homogeneous glycoforms, we have developed an *in-vitro* glycoengineering platform consisting of recombinant Leloir glycosyltransferases expressed in *E. coli*. To demonstrate the applicability of the platform, we have extended paucimannose glycans of Sf9-derived influenza A virus HA (H10N08) to obtain the terminal structure Galα1-3Galβ which corresponds to the α-Gal epitope. Since the anti-Gal antibody is naturally found in humans and constitutes approximately 1% of immunoglobulin G, the α-Gal epitope can be potentially used as a target for developing glycan-based vaccines against different diseases (Galili, U., 2020).
Furthermore, to demonstrate the practical implementation of the platform, his-tagged High Five-derived SARS-CoV-2 spike protein was immobilized on Ni-NTA magnetic agarose beads for *in-vitro* glycoengineering utilizing a set of soluble glycosyltransferases. Galactosylated glycans with Galβ1-4 termina were identified by xCGE-LIF analysis with almost the same proportion compared to the *in-vitro* glycosylated spike obtained without the immobilization protocol. Thus, the possibility to perform the reactions for the *in-vitro* glycoengineering with the immobilized target protein was demonstrated.
The *in-vitro* glycoengineering approach established can be used to efficiently modify a wide range of *N*-glycans on vaccine candidates and therapeutic proteins. To commercialize our technology, we have acquired funding from the EXIST-research transfer program of the Federal Ministry for Economic Affairs and Energy. The project aims to establish a spin-off, eversyn, co-founded by the Max Planck Society by 2024.

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D3) *In vitro* glycan modification of pharmaceutical proteins produced in *Nicotiana benthamiana*

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Pharmaceutical proteins like protein-based vaccines and antibodies are of great importance for an effective treatment of patients. The majority of pharmaceutical proteins are glycosylated and the efficacy of these proteins is in many cases affected by the glycoform attached to the protein. Recombinant protein production systems are able to produce glycosylated proteins, but these systems often produce a wide range of glycoforms of which many lead to a reduced efficacy or no efficacy at all. *Nicotiana benthamiana* has been proven to be a versatile expression system for glycosylated proteins, but has difficulties in generating an exact mimic of the natural eukaryotic glycoforms critical for a high efficacy for the treatment of patients. For example, it is challenging to generate a terminal β1,4-galactosylated N-glycan due to the trimming of plant native galactosidases. *In vitro* modification of these plant produced N-glycosylated proteins can lead to a homogeneous glycosylated protein with the desired glycan structure. In our research, we focus on the heterologous expression of Golgi-localized glycosyl transferase (GT) and glycoside hydrolase (GH) enzymes lacking their insoluble transmembrane region in *Escherichia coli.* These heterologous expressed glycozymes are used in *in vitro* reactions to modify N-glycans of recombinant expressed proteins produced in *Nicotiana benthamiana*, with the goal of generating a pharmaceutical protein with the desired glycan structure.

D4) N-Glycoengineering of *Nicotiana tabacum* BY-2 cells to improve the quality of pharmaceutical proteins

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Recombinant glycoproteins can be produced in various heterologous systems such as insect, mammalian or plant cells. Different N-glycosylation profiles are obtained depending on the cell type and culture conditions. Furthermore, N-glycosylation gives rise to a huge heterogeneity of the final product as each N-site can bear several different N-glycans. This heterogeneity is challenging for pharmaceutical glycoproteins since the presence or absence of N-glycan structures affects their optimal activity.

Nicotiana tabacum Bright Yellow-2 (BY-2) suspension cells is a plant platform that allows growth in bioreactors and protein purification from the culture medium. Our research aims at generating a set of BY-2 cell lines with specific and well-defined glycosylation profiles through targeted knock in and knock out of key enzymes involved in the N-glycosylation pathway.

A BY-2 cell line producing humanized N-glycans was obtained through the inactivation of beta(1,2)-xylosyltransferase and alpha(1,3)-fucosyltransferase (FucT) genes (Mercx et al. 2017). To generate a BY-2 cell line producing glycoproteins with oligomannose N-glycans, the N-acetylglucosaminyltransferase I (GnTI) genes were inactivated (Herman et al. 2021). The N-glycan repertoire analysis of the glycoproteins secreted in those cell lines confirmed the absence of complex N-glycans. Only high-mannose N-glycans, mainly Man4 and Man5, were detected. In addition, a BY-2 cell line expressing glycoproteins with a single N-linked GlcNAc was also generated through the ectopic expression of a fungal endoglycosidase in the GnTI-KO line.

Several glycoproteins, such as antibodies and viral antigens, were successfully produced in the BY-2 glycoengineered cell lines. The N-glycosylation profile of these glycoproteins was characterized and their biochemical properties were analyzed.

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This work was supported by grants from the Service Public de Wallonie in Belgium (WALInnov 1810010).

D5) Efficient bacterial production of mucin-type O-glycoproteins

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The prospect of producing human-like glycoproteins in bacteria is becoming attractive as an alternative to already-established but costly mammalian cell expression systems. We have developed an *Escherichia coli* expression platform that uses a dual-plasmid approach to produce simple mucin-type O-glycoproteins: one plasmid encoding the target protein and the other encoding the O-glycosylation machinery. In combination with genomic integration of the *Neisseria meningitidis neuCAB* operon for CMP-Neu5Ac biosynthesis, we have used this platform to demonstrate high-yielding production of human interferon α-2b, human growth hormone, and a synthetic sequon-optimized peptide bearing mono- and disialylated Core 1 glycans. We further demonstrated that mammalian sialyltransferases, including porcine ST3Gal1, human ST6GalNAc2 and human ST6GalNAc4, are very effective *in vivo* and outperform some bacterial sialyltransferases, including *Campylobacter jejuni* Cst-I and Cst-II. Ultimately, the heterologous expression of mammalian glycosyltransferases in *E. coli* shows promise for the further development of bacterial systems in therapeutic glycoprotein production.

D6) Using glyco-engineered cells with flexible expression of tumor-associated carbohydrates for the generation of highly tumor-specific antibodies

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Background: Highly potent therapeutic approaches require clean targets. However, the majority of antibodies in clinical development or approved for cancer therapy address protein targets that are only overexpressed in cancer and yet often show significant expression in healthy organs. Besides protein expression, glycosylation is strongly altered in cancer, reflecting changes in tumor metabolism or genetic alterations. Mutated or mislocated glycosyltransferases, glycosidases, substrates and chaperones, give rise to tumor-associated, truncated O-glycans like Thomsen-Friedenreich (TF) or Thomsen novelle (Tn) which are expressed on proteins in different carcinomas, leukemias, lymphomas and their metastases.
We have generated a cell line platform which enables tailored immunization and screening approaches in order to generate antibodies against specific protein/carbohydrate combined epitopes (GlycoTargets). These antibodies bind their protein target only in presence of tumor-associated carbohydrates thereby showing markedly reduced binding to the protein expressed in healthy tissues.
Methods: The presence of tumor-associated carbohydrates in cancer tissues was demonstrated by immunohistochemistry analysis. A platform of different glyco-engineered cell lines capable to express proteins with distinct glycoforms was developed and fully characterized. The cells' ability to express GlycoTargets with distinct carbohydrates was shown by flow cytometry, ELISA, and mass spectrometry experiments. Furthermore, the cell line platform was used for recombinant expression of GlycoTargets for the use as immunogens and screening tools in antibody discovery campaigns and lead characterization using ELISA and flow cytometry.
Results: Using our glyco-engineered cells, we showcase the potential of the platform as a tool for the generation of proteins with specific glycosylation and for screening for antibodies that bind to their target protein only in combination with a specific tumor-associated glycan structure. Furthermore, we demonstrate how antibodies are de-selected that cross-react with the non-glycosylated target protein or the glycan itself. This cell-based platform is a proper tool for target validation, antigen production, immunization and screening for highly specific antibodies, opening the field for more effective treatment options.

D7) Synthesis of synthetic lipid-linked oligosaccharides for in-vitro glycoengineering of hemagglutinin peptides by a cell-free, multi-enzyme cascade

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A wide range of peptides can be chemically synthesised or recombinantly expressed in bacteria in an aglycosylated form. To investigate the effect of *N*-glycosylation of these peptides regarding their various functions, i.e. protein stability and receptor binding, an efficient in-vitro *N*-glycosylation system is required.

Here, we present a cell-free system mimicking the Endoplasmic Reticulum glycosylation machinery of eukaryotes. This includes, in particular, the enzymatic transfer of glycans from a lipid-linked (LL) precursor via an oligosaccharyltransferase (OST) onto a peptide. In a first step, a one-pot multi-enzyme cascade employing a set of recombinant glycosyltransferases was established to generate lipid-linked oligosaccharides (LLO) as glycosylation substrates. Using pythanol as a lipid anchor, core-mannose as well as novel hybrid and complex LLOs were generated successfully. In a second step, the single-subunit OST STT3A from *Trypanosoma brucei* was used to transfer these unpurified substrates to the consensus sequence (Asn-X-Ser/Thr) of a synthetic peptide. It is demonstrated that STT3A also shows activity towards simple hybrid structures such as LL-GlcNAc2Man3GlcNAc1Gal1 or the simple complex core-structure LL-GlcNAc2Man3GlcNAc2. Finally, to demonstrate the successful application of the platform to modify polypeptides, we *N*-glycosylated influenza A virus hemagglutinin peptides (HA1) (strain: A/Puerto Rico/8/1934) that were recombinantly expressed as aglycosylated form in *E. coli*. Subsequently, these HA1 peptides were modified using several glycosyltransferases to generate galactosylated and sialylated glycans.

In the future, the developed toolbox will be applied to modify biomedical important peptides with desired glycans to investigate the impact of defined glycosylation on protein function.

D8) Assessing β1,4- & β1,3-galactosidase activity from *Nicotiana benthamiana* leaf galactosidases to enhance galactosylation efficiency

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Plants offer great advantages for the production of glyco-engineered biopharmaceutical glycoproteins, but these glycoproteins are subjected to a wide array of plant-native glycosidases. A consequence of glycosidase activity along the secretory pathway is that the predominant glycoform is steered away from the desired glycan structure. For example, β-galactosidase (BGAL) 1 from the glycosyl hydrolase family GH35 in *Nicotiana benthamiana* has recently been identified as one of the enzymes responsible for preventing efficient β1,4-galactosylation of N-glycans, as well as the ability to cleave mucin-type O-glycans. Strikingly, galactosylation is still not complete in plants lacking BGAL1 activity, indicating that more members of the GH35 family are reducing galactosylation efficiency. In this study, we cloned several novel BGAL open reading frames from *N. benthamiana* leaf material and characterized the biochemical activity of these enzymes. We identified different BGAL enzymes responsible for the cleavage of antennary β1,4-galactose residues of N-glycans on the model glycoprotein kappa-5. Furthermore, we targeted undesired BGAL activity with co-infiltration of a BGAL inhibitor. The identification of additional BGAL enzymes as major targets for efficient engineering of galactose-containing glycans will enable a targeted genome editing approach to reduce undesired processing of these glycans. Effective knockout of these enzymes could allow the production of therapeutically relevant glycoproteins with tailor-made galactosylated glycans in plants.

D9) Tuning the quality of a biopharma product by choice of cell line up to media optimization

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Product titer and quality criteria are the main aspects to be considered in the design of biopharmaceutical protein production processes as each biopharmaceutical has individual properties and requirements. The product quality and thus the potency of a protein may vary greatly from one used cell production system to another. In particular post-translational modifications like glycosylation of biopharmaceuticals can be of crucial importance for bioactivity, efficacy and half-life time. Therefore, the selection of the most suitable expression host is crucial to achieve the desired product characteristics. Here we present the advantage of using different expression platforms, using FyoniBio's mammalian host cell systems CHOnamite® and the human GEX® platform as examples, for the development and production of a certain biopharmaceutical and biosimilars at the desired quality. Two case studies demonstrate the versatility of both expression hosts in product glycosylation and opportunities for bioprocess optimization.

D10) Modelling the processing of glycans on secreted glycoproteins

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N-glycosylation is known to be important for the pharmacological properties and efficacy of many therapeutic proteins (also known as biologics). However, glycosylation is a complex and non-templated process which often results in a highly heterogeneous population of glycan structures, preventing the precise control over the resulting glycan distribution. Therefore, a method to rationally engineer biologics with increased homogeneity and advantageous glycan structures would be highly beneficial to the pharmaceutical industry.

A computational model of glycan biosynthesis, which combines stochastic simulation with Bayesian fitting, has been experimentally validated to provide meaningful insights into the organisation of the glycosylation machinery when simulating the biosynthesis of the total N-glycan repertoire of the cell[1]. The project detailed here aims to use this computational modelling tool to investigate the relationship between organisation of the glycosylation machinery in the Golgi and the glycoform distribution of a model biologic.

We use the modelling to investigate the relationship between whole cell (WC) glycosylation and the glycosylation of the model biologic Herceptin. Glycan profiles from both WC and Herceptin have been acquired in two CHO cell lines: WT and a mutant with altered glycosylation. The computational model predicts how the glycosylation machinery changes between WT and mutant to generate the mutant's altered glycan profile. We will model altered WC glycosylation of the mutant relative to WT, then apply the predicted changes of the glycosylation machinery to the WT Herceptin glycan profile. By assessing how well the Herceptin glycan profile predicted this way matches with experimentally determined one, will allow us to conclude contributions to the glycosylation of a monoclonal antibody therapeutic by both the glycosylation machinery and the protein's structure itself. This information could be used to establish methods to refine computational predictions for controlling biologic glycosylation.

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D11) Effector function evaluation of therapeutic antibodies with defined and homogeneous Fc N-glycans

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N-glycosylation at the fragment crystallizable (Fc) region of immunoglobulin G (IgG) antibodies is a conserved modification of structural and functional importance. Since it impacts antibody-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP), the N-glycan structure is important to take into consideration when developing therapeutic antibodies. However, the structural heterogeneity of the Fc N-glycans complicates analysis and regulation. By combining a chemoenzymatic engineering technology for Fc-specific N-glycan remodeling (Huang *et al*. 2012) and cell-based Fc effector activity bioassays for functional evaluation (Lallemand *et al*. 2017), we here demonstrate fast, specific, and accurate testing.

The technology for generation of homogeneous N-glycans comprises two enzymatic steps starting with Fc-specific deglycosylation to the core GlcNAc, with or without fucose, followed by transglycosylation using oxazoline-activated N-glycans. For the generation of core-afucosylated structures, an exo-α1,6-fucosidase was included during the deglycosylation step. This technology is considerably faster and more efficient than, for example, cellular engineering or cell-free engineering using hydrolases, transferases, and nucleotide sugars.

For effector function evaluation, cell-based reporter gene bioassays utilizing engineered target and effector cells were used. Upon antibody binding to the target cell antigen and effector cell FcγR receptor, a cascade reaction in the effector cell is initialized resulting in luminescence proportional to the functional activity of the antibody. The assays resemble the natural conditions to a greater extent than ligand-based assays, offer less variability than other cell-based assays, and are at the same time fast and accurate, providing easily interpreted results. Here, we generated twelve variants of a therapeutic antibody, each variant homogeneously remodeled with one of the common complex biantennary N-glycan structures with or without core fucose, and evaluated their effector functions. Our data displayed distinct trends regarding core-fucosylation, sialylation, and galactosylation, including differences between the monogalactosylated isomers. Taken together, the use of high-quality systems for antibody N-glycan remodeling and effector function evaluation will gain valuable knowledge about the relationships between Fc N-glycosylation and effector functions, and facilitate such analyses during monoclonal antibody development.

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D12) Influence of peptones on CHO glycosylation profiles

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Efforts in upstream process improvement primarily focus on optimizing CHO cell growth and protein production. However, appropriate glycosylation of monoclonal antibodies (mAb) is critical for their safety and efficacy. Modification of glycosylation profiles can affect serum half-life, recruitment of immune effector cells, stability, cytotoxicity, anti-inflammatory properties, and antigen affinity. Glycosylation patterns vary amongst different host cell lines and can further be influenced by upstream process parameters, including dissolved oxygen levels, culture temperature, pH, and nutrient availability. For instance, a limitation of glutamine or glucose can lead to changes in sialylation, as well as hybrid and high-mannose glycans. Here, we investigated how nutrient availability and complexity affect glycosylation profiles of mAb produced with CHO-K1, CHO-K1 GS, and DG44 cell lines. Evaluation of different chemically defined cell culture media and feeds led to minimal changes. Addition of plant-derived peptones led to pronounced alterations in mAb glycosylation, enabling modulation of glycan structures to achieve a desired biosimilar profile. While supplementation with soy peptones led to an increase in more differentiated glycans and decrease in acidic charge variants, addition of cotton and wheat peptones increased less-differentiated glycans and did not alter the charge variant profile. Thus, targeted supplementation of CHO cell cultures with peptones can improve productivity and glycosylation profiles.

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Tools & Technologies for Glycoanalytics and Glycobioninformatics

E1) Novel application of MXene for N-glycan enrichment

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Over the last years the study of sialic acid linkages has become interesting as a promising biomarker for cancer diagnostic since α2,6- and α2,3-sialylation have been associated with the development of cancer, tumor growth, and metastasis (Zhou et al, 2020). Due to sialic acids are very labile, low ionization efficiency, complexity especially the glycosidic linkages, heterogeneity and, its low abundance in the human glycome make them very challenging to analyze by Mass Spectrometry (MS). There are many approaches available such as permethylation, amidation and esterification to enhance ionization efficiency in MS positive mode. A detailed MS analysis of sialylated glycans normally requires high purities, costly clean-up procedures, and chromatographic separation techniques. Therefore, protocols for glycan enrichment are needed to find out clinically relevant sialylated glycan biomarkers.

MXenes, belongs to the new generation of two-dimensional (2D) nanomaterial, presenting exceptional physico-chemical properties such us hydrophilicity, large surface area and, electrical conductivity. MXenes nanosheets are synthesized from MAX phases represented by $M_{n+1}X_nT_x$ formula, where, “M” is an early transition metal, “X” represents C or N, and “T” represents the terminal group (−F, −OH, and =O). These groups provide highly active and surface sites with unique properties making MXene promising hydrophilic stationary phase for *N*-glycan enrichment (Aguedo et al, 2020).

In our study we described for the first time the application of MXene for *N*-glycan enrichment in combination with chemical derivatization strategy and analyzed by MALDI-TOF MS allowed us to differentiate isoforms of sialylated glycans for further applications in glycan-based biomarker discovery.

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E2) Rapid High-Throughput Profiling and Quantitation of Sialic Acids in Biotherapeutics

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The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics and pharmacodynamics (Liu, L. 2015). Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of therapeutic glycoproteins (Varki, A. 2008). Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory (Li, Y. et al 2017; Scallon, B. J. et al 2007; Kaneko, Y. et al 2006). Two forms of sialic acid commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycoylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to study not only the absolute quantity of sialic acid, but also the levels of different sialic acid species present in therapeutic glycoproteins.

Here we present a new high-throughput workflow based on a 96-well plate format for the release, labeling, and analysis of sialic acids from therapeutic glycoproteins using rituximab, etanercept, and NISTmAb as examples. Sialic acid residues are released then labeled with 1,2-diamino-4,5- methylenedioxybenzene (DMB) in a two-step procedure. The DMB-labeled sialic acids are then separated and analyzed using a rapid 10-minute method based on reversed-phase ultra high-performance liquid chromatography (UHPLC) coupled with fluorescence and optional mass spectrometry detection. The workflow offers both qualitative characterization of Neu5Ac, Neu5Gc and other sialic acid species using a sialic acid reference panel (SARP), as well as absolute quantitation with picomolar level sensitivity using included Neu5Ac and Neu5Gc quantitative standards. The workflow enables reliable and reproducible high-throughput profiling and quantitation of sialic acids, providing a broad detection range and improved sensitivity for molecules with low levels of sialylation.

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E3) Enzymatic Degradation of Interfering Oligosaccharide Impurities for Reliable *N*-Glycan Analysis

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Reliable *N*-glycan analysis is of great importance for a variety of disciplines in science, medicine, and industry. The challenging analysis caused by the sheer diversity of *N*-glycans gets even more complicated by occasional oligosaccharide impurities (OSIs). These typically comprise linear glucose homopolymers with α(1-4)- (maltodextrins) or α(1-6)-linkages (dextrans). As OSIs have similar physicochemical properties as *N*-glycans, measurement signals of OSIs and *N*-glycans can overlap when analyzed by liquid chromatography, mass spectrometry or capillary electrophoresis. This complicates *N*-glycan identification and can even lead to structural misinterpretations. If recognized, OSI signals are often excluded in data evaluation, which potentially impairs results by accidentally neglecting valuable *N*-glycan signals. Alternatively, a washout of OSIs from glycoprotein samples before *N*-glycan release could be included into a repeated sample preparation. However, this increases the effort, time, and sample amount required, and a successful OSI removal is not guaranteed.

To overcome these issues, we investigated the glucoside hydrolase-supported degradation of OSIs in the fully processed *N*-glycan samples. Therefore, we screened ten commercially available enzymes concerning their potential to degrade interfering maltodextrins and dextrans in *N*-glycan samples. Enzyme reactions were carefully monitored by multiplexed capillary gel electrophoresis with laser-induced fluorescence (xCGE-LIF). Only four of the tested enzymes allowed a fast and effective degradation of maltodextrins or dextrans. Since all these enzymes were not originally intended for *N*-glycoanalysis, we tested the four candidates regarding side activities with *N*-glycans derived from bovine IgG, fetuin and ribonuclease B. As expected, some of the tested enzymes exhibited β-galactosidase, *N*-acetylglucosaminidase and/or α-mannosidase side activities, rendering them unsuitable for the intended application. These results clearly suggest that utmost care must be taken when selecting and using enzymes for reliable glycoanalytics. Finally, we demonstrate a side-reaction-free and effective degradation of OSIs in fully prepared *N*-glycan samples by incubation with the two remaining enzymes (dextranase from *Chaetomium erraticum* or glucoamylase P from *Hormoconis resinae)* for only 30 min.

Overall, as OSIs do not always display typical and easily recognizable ladder patterns, we suggest including a glucoside hydrolase treatment in routine *N*-glycan analysis.

E4) Quantitative exploration of beam-type glycopeptide fragmentation using stable isotope-labeled standards

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Mass spectrometry (MS) is one of the most powerful tools for the analysis of glycoproteins and their glycoform structures. Collision-induced dissociation (CID) is the most common fragmentation used in LC-MS/MS analysis of glycopeptides. Collision energy (CE) is a critical parameter that can be used to improve structural resolution, as different linkages of glycan units show different stabilities to CID/HCD fragmentation. Quantitative and qualitative resolution of glycopeptide isobaric structures is one of the biggest challenges of glycoproteomics measurement. Fragmentation of the glycan moiety produces a low molecular weight ion (oxonium ions) that can serve as a structure-specific signature for specific structures such as LacdiNAc, polyLacNAc, outer arm fucosylation or SialoGlcNAc. Here, we report the use of CE modulation in combination with stable isotope-labeled glycopeptide standards at the reducing terminal GlcNAc to investigate the production of outer antennary structure specific oxonium ions. For example, LacdiNAc ion could be produced by fragmentation of the outer antenna structure LacdiNAc (GalNAc-GlcNAc) as well as by fragmentation of the core of chitobiose (GlcNAc-GlcNAc). With terminal GlcNAc labelling, we are able to resolve diHexNAc with different origins. In addition, we are able to quantitatively describe the production of a false-positive LacNAc-fucose ion caused by fucose rearrangement. This work can significantly improve glycopeptide identification and structural resolution confidence by providing additional information to established glycopeptide search algorithms and tools.

E5) Integrating dynamic simulation within GlycoSim for the quantitative analysis of glycosylation pathways

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In the last few decades, several research efforts have been undertaken to develop mathematical models of glycosylation, primarily with the aim to predict the glycoprofiles of protein-based biotherapeutics and to provide insight into the glycan biosynthetic machinery itself (Shek et al 2021). However, while effectively predicting the relative abundance of desired glycans in their respective domains, their wide adoption and utilisation from the glycobiology community remains elusive. This can be partly attributed to the high level of expert domain knowledge required to model biological systems and to the limited accessibility to such models. Thus, the quantitative analysis of glycosylation pathways, both in clinical and biomanufacturing settings, would greatly benefit from integrating existing mechanistic models of glycosylation within publicly available bioinformatic tools. This work presents the integration of dynamic simulation within GlycoSim (https://glycosim.rings.glycoinfo.org), an existing online simulation tool that belongs to the RINGS platform (Akune et al 2010). Following a systems biology approach, dynamic simulation within GlycoSim is underpinned by a previously published mathematical model of Golgi processing (del Val et al 2011), which was adapted to run dynamically for a user-defined simulation period. This allows the user to observe the evolution of glycan concentrations over different time spans, thus providing the flexibility to capture the dynamics of the Golgi. As a result, GlycoSim currently supports multiple interconnected functionalities, including (1) the integration of an arbitrarily complex *in silico* generated glycosylation reaction network based on a fixed enzyme set using existing RINGS tools (Glycan Pathway Predictor) into the dynamic model; (2) the description of the enzyme kinetics of this network; (3) the estimation of enzyme and nucleotide sugar transport protein levels for a given dataset using a hybrid stochastic and deterministic optimisation approach; (4) the performance of dynamic simulation using the information from (2) and (3). Finally, dynamic simulation can be tailored to specific glycoproteins, which enables the user to integrate various protein-specific glycomic datasets. We envisage that GlycoSim will facilitate the quantitative analysis of glycosylation pathways by glycobiologists via a publicly available web-based interface without the need of prior modelling and bioinformatic knowledge and experience.

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E6) LC-MS/MS based Subcellular Quantification of Sugar Nucleotide precursors in CHO secretory pathway

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One of the most important features of glycoproteins such as antibodies is their glycosylation pattern, which defines stability, immunogenicity, and residence time in the blood.
In order to gain a deeper mechanistic understanding of how cultivation conditions affect antibody glycosylation, we developed an integrative analytical workflow for reliable quantification of subcellular concentrations of nucleotide sugars in the cytosol and Golgi lumina, allowing for an empirical consideration of eukaryotic compartmentalization. The method is based on cell fractionation via Digitonin (Holden 2009) and exhaustive sample extraction followed by LC-MS/MS analyses maintaining the physiological context of absolute metabolite pools (Feith 2019). Information obtained will potentially help to create more precise glycosylation models in the future by empirically determining precursor concentrations within the organelles of their consumption.
Ongoing work focuses on the transfer of the approach to CHO cultivations to elucidate subcellular precursor dynamics.

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E7) Clinical glycomics for the diagnosis of congenital disorders of glycosylation

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Congenital disorders of glycosylation (CDG) refer to a heterogeneous group of diseases caused by inborn defects in various steps along *N*- and *O*-glycosylation pathways. Because the symptoms caused by CDGs are rather unspecific, clinical diagnosis of this disease group is difficult. Often, genome sequencing by next-generation sequencing technologies is required to detect genetic defects of glycosylation-linked enzymes for diagnosis.
To overcome these issues, we present ways to fast and reliably detect CDGs by multiplexed capillary gelelectrophoresis with laser-induced fluorescence detection (xCGE-LIF) with only one droplet of blood. In particular, we demonstrate the potential of the method on a female patient bearing a CDG manifested by mutations of the mannosyl-oligosaccharide glucosidase (MOGS) gene. This defect results in a decreased activity of the enzyme glucosidase I, causing a systemic change in *N*-glycosylation. The straightforward xCGE-LIF-based glycoprofiling analysis revealed an aberrant *N*-glycosylation, which is exclusively present in the patient, but not in healthy relatives. The aberrant *N*-glycan precursors $Glc_3Man_{7\text{-}9}GlcNAc_2$ could be found inside the patient's blood serum and on serum derived proteins like IgG. Furthermore, the accumulation of the free tetrasaccharide *Glc(α1-2)Glc(α1-3)Glc(α1-3)Man* could be observed in the patient´s blood. This tetrasaccharide is the product of an alternative bypass of the glucosidase I dependent *N*-glycan processing via endo-α-1,2-mannosidase. Detection of this tetrasaccharide using xCGE-LIF might in the future serve as a biomarker as part of patient screening during diagnostic work-up.

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E8) Challenges in analysing complex N-glycan patterns of biopharmaceuticals

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A classical approach for N-glycan analysis is composed of enzymatic digest and release, labelling and chromatographic separation. Antibodies N-glycans consist typically of ~ up to 10 known major N-glycan structures (Liu, 2015). A first annotation is usually done by using single N-glycans or a well characterized N-glycan mixture derived from an antibody (e.g. NIST-standard (Prien et al, 2015)) as a standard. Analyzing N-glycan patterns of enzymes is challenging since its composition is quite often high in diversity of less characterized and complex N-glycan structures. Phosphorylated species are one of those less characterized N-glycans that might be present and might be a critical quality attribute e.g., in case of being essential for the cellular uptake of a biopharmaceutical into the lysosome (Kang et al, 2021).
The low recovery and low reproducibility of low abundant phosphorylated N-glycans is challenging. Low abundancy is most likely caused by phosphate/stainless steel interactions via phosphate/iron complexes within the HPLC system (Pat et al, 2018).
This poster presents the analysis of a challenging complex N-Glycan pattern of a biopharmaceutical and its optimization. Firstly, the optimization of the N-glycan analysis (relative abundances), secondly the optimization of the Mannose-6-Phosphate (M6P) analysis followed by the absolute quantification of the M6P content are demonstrated. For the quantification of M6P an innovative strategy was chosen. Monosaccharide analysis is commonly performed using a HPAEC-PAD, RP-HPLC or a GC-HPLC approach: it was focused on quantification by hydrophilic interaction chromatography (HILIC). Sample preparation included the following steps: (i) acidic hydrolysis, (ii) labelling and (iii) HILIC-HPLC-FLD analysis. System interactions are avoided by the use of citric acid as metal complexing mobile phase additive to reduce the metal ion-mediated adsorption (Hsiao et al, 2018). The presented method uses a HILIC and MS compatible mobile phase additive eluent system, which is robust and ready for validation within the GMP environment.

Abbreviations

FLD	Fluorescence detector
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
M6P	Mannose-6-phosphate
RP-HPLC	Reversed phase high performance liquid chromatography

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E9) High-throughput LC-MS based analysis of plasma protein glycosylation

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High-throughput glycomics gained a momentum in the last 15 years with the development of the methods adapted and optimized for the 96-well format (Ruhaak et al 2008; Royle et al 2008; Ruhaak et al 2010). Analytical processes were optimized on human plasma as major sample for the analysis primarily having glycans as new potential biomarkers in the focus of the research. Since then number of different methods were developed and optimized on different analytical platforms covering liquid chromatography with fluorescent detection, capillary electrophoresis with fluorescent detection or hyphenated to mass spectrometry, mass spectrometry based analytical approaches both with and without LC separation and lectin arrays (Trbojević-Akmačić et al 2022). In the last couple of years in our lab we introduced number of analytical techniques covering several plasma proteins, both human and rodent. Immunoglobulin G as the major analyte isolated from human, mouse and rat plasma, analysed on released glycan level and on the site-specific N-glycosylation level using UHPLC-FLR and LC-MS, respectively (Habazin et al 2021). Just recently, analytical approaches for two additional human plasma proteins, enriched to a higher level but not completely purified, were introduced for the analysis of glycopeptides. Methods for the Alpha-1-acid glycoprotein (AGP) and Complement component 3 (C3) site-specific N-glycosylation LC-MS analysis were proven on several large cohorts as qualitative analytical methods capable of detecting biologically meaningful data (Keser et al 2021; Šoić et al 2022). Glycosylation is one of the essential factors for the functioning of every organism and the development of effective analytical methods for qualitative and quantitative detection of glycans and their changes under various physiological and pathological conditions is a prerequisite for the successful research and introduction of glycans as clinically relevant biomarkers.

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E10) GlycoSHIELD: an online tool to address glycan dynamics and heterogeneity in glycoproteins

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The majority of membrane and secreted proteins are post-translationally modified through a covalent addition of complex sugars: glycans. Glycans influence protein-protein interactions and can be hijacked by pathogens like the SARS-CoV-2 virus to sidestep the immune system. The hydrophilic character and lack of secondary structure result in large conformational freedom of glycans, which, together with glycan microheterogeneity, hinder the complete structural characterisation of glycoproteins. Molecular dynamics simulations have been instrumental in addressing glycan dynamics and function, but are impractical to study large protein complexes or to elucidate the effects of various glycoforms due to the required computing time. Taking advantage of the disparity of the time scales of conformational changes of glycans and proteins, we propose a reductionist open-source method to graft ensembles of glycan conformers onto static protein structures: GlycoSHIELD. Through comparisons with extended atomistic simulations of the SARS-CoV-2 spike glycoprotein, we demonstrate that GlycoSHIELD reproduces key features of the shielding effect of glycans at a fraction of the computational cost. Furthermore, we show that reconstructed glycans can be used to complement existing cryoEM structures of glycoproteins. GlycoSHIELD, together with a library of 46 glycan types to date is available for non-expert users at www.glycoshield.eu.

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E11) New avenues for biomarker discovery in human blood plasma via an improved in-depth analysis of the low-abundant *N*-glycoproteome

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To understand the molecular implications of protein glycosylation and to leverage those insights for clinical diagnostic and biopharmaceutical production innovative glycoproteomic technologies are required (Kirwan et al 2015; Zhou et al 2019). Essential elements in this regard are the site-specific identification, structural characterization, and quantification of the protein glycosylation. Recently significant advances were made particularly towards structure-focused *N*-glycoproteomic analyses. The mass spectrometric analysis of intact *N*-glycopeptides using stepped collision fragmentation along with glycan oxonium ion profiling, for instance, now enables to reliably discriminate between different *N*-glycan types and to discern isobaric structural features such as antenna- and core-fucosylation (Hoffmann et al 2018).
Still, there are some weak points current *N*-glycoproteomic approaches are affected by. Among the most significant are: 1) handling of incorrect identifications 2) identification of rare and modified *N*-glycans such as sulfated *N*-glycans 3) insufficient glycoproteomic coverage particularly in complex samples such as human blood plasma.
To address these shortcomings, we have developed an innovative *N*-glycoproteomic workflow that aims at providing comprehensive site-specific and structural *N*-glycoproteomic data on human blood plasma glycoproteins – primarily derived from the middle and lower concentration range. To achieve this, the workflow features protein enrichment and fractionation strategies, the use of high-resolution mass spectrometry with stepped collisional energy fragmentation, and an extended set of marker ions that were included into the glycan oxonium ion profiling. In addition, the workflow also covers a new data validation strategy by including a semi-automated decision tree procedure.
With this workflow we were able to advance in the analysis of the human blood plasma *N*-glyco-proteome by being able to analyze human blood plasma glycoproteins that have concentrations as low as 101 pg/mL (Nanjappa et al 2014). Furthermore, we could significantly improve the description of the *N*-glycan micro-heterogeneity by including rare *N*-glycans such as sulfated and glucuronidated ones. Our analysis also includes the confident differentiation of ambiguous *N*-glycan structures like bisecting GlcNAc or antenna- vs core-fucosylation. In total 1929 *N*-glycopeptides and 942 *N*-glycosites derived from 805 human middle to low abundant glycoproteins were identified.
Overall, the presented workflow holds great potential to increase our understanding of protein glycosylation and to foster the discovery of blood plasma biomarker.

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E12) Entering the N-glycan structure jungle from two sides

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The enormous structural diversity accomplished by just a few building blocks and enzymes poses a huge analytical challenge. Simple systems such as IgG can be tackled with basically "one-dimensional" methods such as HILIC-HPLC with fluorescence or mass spectrometric (MS) detection. Choice of a shape-selective stationary phase, *i.e.* porous graphitic carbon (PGC), coupled to MS provides superior isomer separation. Individual peaks are characterized by their (negative mode) fragment spectrum and their retention time – the latter, however, is prone to some fluctuation. We propose normalizing retention times with a tightly spaced Time Grid (glyco-TiGr) of isotope-labeled internal standards. Using recombinantly expressed glycosyltransferases, a library of normalized retention times covering around 140 structures has so far been built. Of that, not less than 40 isomeric structures consisting of five hexoses, four *N*-acetylhexosamines and one fucose residue were generated. Notably, this endeavour resulted in the detection of novel bisected N-glycans in brain (Helm et al, 2022; Helm et al, 2021).
Predictably, even the most skillful application of PGC-LC-MS will become entangled in the impenetrable "jungle" of fucosylated and sialylated tri- and tetraantennary structures. Irrespective of advances in separation science, isomeric large glycans will not be sufficiently separable to allow for unambiguous identification by retention time or/and negative mode MS/MS. Even worse, the results of glycomic studies usually take the shape of exceedingly long lists that are almost incomprehensible to the human mind. A remedy to this problem could be found in the comprehensive characterization of critical glycan epitopes by breaking down large glycans into smaller elements of limited diversity. Chemical degradation of a sample „distills" the almost infinite number of distinct glycan structures of a given N-glycome to a tractable number of smaller oligosaccharide fragments, which are unambiguously identified by their masses and specific retention times on PGC. Thus, even highly complex glycan mixtures can be characterized by a comprehensible glycan-feature foot print.

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E13) New approach using Online SPE purification and procainamide for HILIC-FLR-MS N-glycan analysis

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Glycosylation is one of the most prominent post-translational modification of proteins that plays a crucial role in maintenance of the structure and protein activity. In the last decades, monoclonal antibodies (mAbs) continue to reign supreme the biopharmaceutical approvals due to their successful treatment of a vast array of serious diseases, such as cancers, immune disorders, and infections. mAbs are glycoproteins and the modifications in the monosaccharide residues may alter their functioning such as their half-life, immunogenicity, toxicity, stability, and solubility. Therefore, special attention should be paid to the detection of changes in the glycosylation patterns. The main goal of our study was to develop and validate a rapid sample preparation using procainamide labelling and online SPE purification to improve monitoring of N-glycans and compare the procedure with other commercial kits. This analytical approach will be used to monitor batch to batch sample of glycosylated biotherapeutics but also to investigate and to understand the impact of N-glycosylation alteration at several diseases like endometrioses and human immunodeficiency virus (HIV).
Briefly, N-glycans were (i) enzymatically released using PNGase F, (ii) labelled with procainamide or commercial kits: GlycoWorks RapiFluor-MS N-Glycan Kit[1] (RFMS kit) or Instant PC Kit, (iii) cleaned with µHILIC SPE plate or online HILIC SPE purification and (iiii) analyzed by HILIC coupled to a high-resolution mass spectrometer detector and a fluorescence detector.
Comparing the two approaches (online HILIC SPE purification and on the bench, purification using the µHILIC SPE plate) we proved that the online purification offers a higher sensitivity and abundance for the released N-glycans beside the better repeatability and the time saving. All these advantages came with accuracy and cost-effective.. Our protocol showed a higher sensitivity for the MS signals with a slight lower FLD signals comparing to the RFMS and the instant PC. We also tested the NIST mAb standard to compare our method to published results and we obtained the same observed ratio of galactosylation, sialylation, and fucosylation than described in literature. This protocol was actually used to characterize batch to batch variation of biopharmaceutical (Trixuma) and also applied to investigate N-glycans modification in endometrioses.
The described approach offers a reliable and repeatable method for released N-glycans analysis with a high sensitivity and a cost-effective manner. It showed its efficiency in analysis of biosimilars and antibodies from clinical samples

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E14) Methodical approach to design and optimize microarrays for efficient and high-throughput glycoprofiling and application in cancer sera glycoprofiling

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Glycosylation being a canonical posttranslational modification in eukaryotes is a key cellular mechanism regulating several physiological and pathological functions. Microarray has been the sole analytical platform, since their development in the past two decades, for analyzing carbohydrate-mediated recognition events in a high-throughput manner. Depending on the configuration i.e., lectin or glycan/glycoptotein which is immobilized on the slide surface, microarrays for glycomics studies can be broadly categorized as lectin and glycan/glycoprotein arrays respectively. Glycan/glycoprotein arrays have been vastly adopted to determine glycans specificities of glycan-binding proteins (GBPs) or glycoprofile samples in a high-throughput manner principally using GBPs. Each microarray consists of a large number of distinct glycans or glycoprotein samples immobilized on a solid substrate in well-localized discrete spots. Identification of presented glycan structures is performed by incubating with probes, mostly lectins, able to identify specific glycan moieties. However, several factors influence the final readout of these binding events ranging from concentration of the samples, spotting parameters, incubation parameters as well as the scanner parameters like pixel size, scan speed and most importantly the voltage applied to the photomultiplier tubes (PMT). Here we demonstrate the influence of some of these parameters on the results of the binding events with a goal to find appropriate parameters for performing reliable glycan/glycoprotein microarray analysis. The optimized microarray procedures were then applied in glycoprofiling of serum and depleted serum samples of more than 200 patients diagnosed with different types of cancer as well as for glycoprofiling of patients' serum samples diagnosed certain types of cancer before and after cancer treatment.

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